PHYSIOLOGICAL STUDIES ON A NONSYMBIOTIC PLANT HAEMOGLOBIN IN A TRANSGENIC MAIZE CELL SYSTEM

By

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To Dorota, I thank you for being there.

I dedicate this thesis to my Mother and Father, and to Dorota.

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List of Abbreviations

AA	Antimycin A
ADH	Alcohol dehydrogenase
Adh	Alcohol dehydrogenase gene
ADP	Adenosine 5'-diphosphate
ANP	Anaerobic polypeptide
ATP	Adenosine 5'-triphosphate
BMS	Black Mexican Sweet (Maize cultivar)
dCTP	deoxycytidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetoc acid
EGTA	Ethylene glycol-bis-N,N,N',N'-tetraacetic acid
g	gram
h	hours
Hb	Haemoglobin
kD	kilodaltons
kb	kilobase pairs
Lb	Leghaemoglobin
LDH	Lactate dehydrogenase
Ldh	Lactate dehydrogenase gene
M	Mole
Mb	Myoglobin
min	Minutes

mg	miligram
ml	mililiter
NADH	Nicotinamide adenine dinucleotide (reduced)
μg	microgram
μl	microliter
PAGE	Polyacrylamide gel electrophoresis
PDC	Pyruvate dehydrogenase
SDS	Sodium dodecyl sulfate
VHb	*Vitreoscilla* haemoglobin
vgb	*Vitreoscilla* haemoglobin gene

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ABSTRACT

Nonsymbiotic haemoglobins are broadly present across the plant kingdom, however, the function of these proteins is unknown. Cultured maize cells have been transformed to constitutively express a barley haemoglobin gene in either the sense (HB^+) or antisense (HB^-) orientation. Haemoglobin protein expression in the transformed cell lines was correspondingly higher or lower than in wild type cells under normal atmospheric conditions. Limiting oxygen availability by placing the cells in a nitrogen atmosphere for 12 hours had little effect on the energy status of cells constitutively expressing haemoglobin, but had a pronounced effect on both wild type and HB^- cells, where ATP levels declined by 27% and 61% respectively. Total adenylates in these cells were approximately 35% lower than in HB^+ cells. Energy charge was relatively unaffected by the treatment in HB^+ and wild type cells, but was reduced from 0.91 to 0.73 in HB^- cells suggesting that the latter were incapable of maintaining their energy status under the low oxygen regime. Treatment of the cells grown in an air atmosphere with electron transport inhibitor, antimycin A gave essentially the same results. The presence of haemoglobin enhanced oxygen uptake by the cells under conditions of low oxygen availability. It is suggested that nonsymbiotic haemoglobins act in plants to maintain the energy status of cells in low oxygen environments and that they accomplish this effect by promoting glycolytic flux through NADH oxidation, resulting in increased substrate level phosphorylation. Hypoxic acclimation of plants is an example of this effect in nature. Nonsymbiotic haemoglobins are likely ancestors of an early form of haemoglobin that

sequestered oxygen in low oxygen environments, providing a source of oxygen to oxidize NADH to provide ATP for cell growth and development.

I. OBJECTIVES

Haemoglobins are wide spread throughout the biosphere. They are found in a broad range of organisms from bacteria, through unicellular eucaryotes, to plants and animals suggesting that they predate divergence of life into plant and animal forms. Plant haemoglobins have been classified into symbiotic and nonsymbiotic types. Symbiotic haemoglobins are found in plants that are capable of participating in microbial symbioses, where they function in regulating oxygen supply to nitrogen fixing bacteria. Nonsymbiotic haemoglobins have only recently been discovered and are thought to be evolutionary predecessors of the more specialized symbiotic leghaemoglobins. The ubiquitous nature of nonsymbiotic haemoglobins is evidenced by their broad presence across the plant kingdom. The widespread presence and long evolutionary history of plant haemoglobins suggest a major role for them in the life of plants. Very little , however, is known about their function, although it has been proposed that nonsymbiotic haemoglobins may act either as oxygen carriers to facilitate oxygen diffusion, or oxygen sensors to regulate expression of anaerobic proteins during periods of low oxygen supply. Recent findings of high oxygen avidity of the nonsymbiotic haemoglobins may, however, seriously question validity of these hypotheses.

The main objective of the research presented in this thesis is , therefore, to add to the understanding of the physiological significance of the nonsymbiotic haemoglobins in plants. A common approach to the studies of the function of a protein is that some useful information may by gained from the observation of its expression pattern. In fact, it was

noted that the expression of some nonsymbiotic haemoglobins from prokaryotic and eucaryotic sources, including plants, is induced by oxygen deficit. This may suggest that these proteins are part of organism's strategy of survival under the conditions of limiting oxygen.

This thesis takes an alternative approach to the search for the function of the nonsymbiotic haemoglobins in plants by examining the effects of its expression. A perspective taken by the research presented here is that the effects of haemoglobin presence may be emphasized by genetically altering the ability of plant cells to synthesize Hb. It is, therefore, essential to create a study system consisting of transformants with increased expression of haemoglobin through a transformation with a haemoglobin gene under the control of a strong constitutive promoter, and with decreased ability to synthesize Hb through a transformation with an antisense Hb gene.

Since the barley haemoglobin cDNA will be used in the studies, a plant material must be identified that possesses a Hb gene sharing enough homology with the barley Hb to grant cross-hybridization for detection and the antisense RNA effect, and whose Hb expression is hypoxia inducible in a manner similar to that of barley. It is also essential that the plant material used in the studies is amenable to genetic transformation. Transformation vectors should be constructed with the full length barley Hb cDNA in the sense and antisense orientations under the control of a strong constitutive promoter. Transformants should be selected for further studies on the basis of their Hb expression.

A comparison of physiological characteristics of the transformants with the increased and decreased Hb expression as well as the wild type plant cells, with the

emphasis on their performance under limiting oxygen conditions, may reveal some effects that are mediated by the Hb. These in turn, may serve as clues for uncovering the physiological significance of the nonsymbiotic haemoglobin in plants.

II. LITERATURE REVIEW

INTRODUCTION

In this review some general aspects regarding the presence of nonsymbiotic haemoglobin in plants will be presented with the emphasis on those related to its function. The differences between the symbiotic and nonsymbiotic haemoglobins will be pointed out to demonstrate that both constitute distinct classes of globins, in terms of sequence and properties.

As it becomes apparent that the nonsymbiotic haemoglobins are widespread across the plant kingdom and that they represent a more primitive and evolutionary older form of the plant globin genes, the question of their function becomes more attractive. While the physiological functions of the symbiotic haemoglobins in plants are well understood, almost nothing is known about their nonsymbiotic predecessors. Therefore, the known and hypothetical functions of haemoglobins in various systems will be described along with information concerning properties and the regulation of expression of the nonsymbiotic haemoglobins, with the hope that some valuable information might be deduced.

Interestingly, a number of nonsymbiotic haemoglobins have been shown to be hypoxia-inducible. The spatial and temporal pattern of this induction in barley may suggest that it is an integral part of the plant's response to limiting oxygen stress. With this in mind, plant responses to hypoxia and anoxia will be reviewed, focussing on the energy metabolism that is most severely affected under such stresses.

A perspective taken by this thesis is that physiological effects mediated by

haemoglobin might be demonstrated through genetic manipulations of its expression in an artificial, transgenic system. Methods of plant cell transformation will be, therefore, presented. Advances in plant transformation technology have made it possible to achieve over expression as well as inhibition of the expression of a given gene in a plant cell. Antisense RNA-mediated gene silencing and the problems of cosuppression will be briefly discussed.

WHAT ARE HAEMOGLOBINS?

Haemoglobins are characterized by their ability to reversibly bind oxygen. A functional haemoglobin consists of a globin polypeptide and the prosthetic group, haem, that is non-covalently bound to the globin. Haem is a tetra pyrrole ring, protoporphyrin IX, with a chelated iron atom and it is the oxygen binding site of haemoglobin (Voet and Voet, 1990). Haemoglobins are relatively small, globular molecules with molecular weights clustered around 18,000 (Wittenberg and Wittenberg, 1990). In nature, haemoglobins exist in monomeric, dimeric, or tetrameric forms. The red blood cell haemoglobins of vertebrates, for example, are heterotetramers built with two α and two β subunits (Perutz *et al.*, 1978). Plant haemoglobins are either homodimers without haem-haem interaction or monomers (Appleby, 1992; Duff *et al.*, 1997). The three - dimensional structure of haemoglobins or their subunits is best represented by myoglobin, a monomeric haemoglobin of vertebrate muscle cells (see Rawn, 1994 for the stereoscopic models of myoglobin and other haemoglobins).

Oxygen binding can occur only if the haem iron is in the Fe^{2+} (ferrous) valence state and the hydrophobic environment in the protein cleft, provided by valine and phenylalanine residues, minimizes the oxidation rate of Fe^{2+} (Appleby, 1992; Rawn, 1994). The interaction between the polypeptide and the haem affects the kinetics of oxygen binding by haemoglobins, therefore, haemoglobins with different amino acid sequences differ in their affinity for oxygen. It will be shown, in this review, that haemoglobins have evolved to perform various functions, reflected by their different ligand binding kinetics (Table 1).

Table 1. ***Kinetics and equilibrium constants for the reactions of various haemoglobins with oxygen***

Protein	k'_{on}	k_{off}	K_D (=k:k')	Reference
	$\mu M^{-1}sec^{-1}$	s^{-1}	nM	
Ascaris Hb	1.5	0.004	2.7	Gibson and Smith, 1965
Barley Hb	9.5	0.0272	2.86	Duff *et al.*, 1997
Rice Hb1	68	0.038	0.5	Arredondo-Peter *et al.*, 1997
Arabidopsis Hb1	75	0.12	1.6	Trevaskis *et al.*, 1997
Arabidopsis Hb2	1.1	0.14	130	Trevaskis *et al.*, 1997
Parasponia Hb1	165	15	89	Gibson *et al.*, 1989
Casuarina symHb1	41	6	135	Gibson *et al.*, 1989
Soybean Lb	120	5.6	48	Gibson *et al.*, 1989
Sperm Whale Mb	14	12	875	Springer *et al.*, 1986
Vitreoscilla Hb			6000	Dikshit& Webster, 1988

The equilibrium dissociation constant (K_D) represents the dissolved oxygen concentration at which the protein is half combined with ligand.

Oxygen affinity of haemoglobins can be greatly affected by environmental factors, such as pH, CO_2 and organic phosphate (Komiyama *et al.*, 1995; Giardina *et al.*, 1995). Concentration of haemoglobin itself can also be a factor affecting its ligand binding (Airaksinen and Nikinmaa, 1995).

Certain small molecules, such as CO, NO and H_2S, bind to the sixth liganding position of the Fe(II) in haemoglobin with much higher affinity than does O_2. This as well as their similar binding to the haem of cytochromes accounts for their highly toxic properties (Voet and Voet, 1990).

In haemoglobins that are built from more than one subunit, such as tetrameric haemoglobins of vertebrates, haem - haem interactions affect oxygen binding. This interactive binding phenomenon is called cooperativity (Rawn, 1994). Binding of one oxygen molecule to one haem facilitates binding of oxygen to the remaining haems within the same haemoglobin molecule.

A consequence of reversible combination with oxygen are two different forms of haemoglobin: oxygenated and deoxygenated. Binding of oxygen, or any other ligand, changes conformation of the protein. The functional implications of this change will be discussed later in this review.

A distinct characteristic of haemoglobin, as well as other haemoproteins, is its red colour brought about by the presence of haem. The oxy-haemoglobin shows two absorbance peaks in the visual spectrum, at about 540 and 574 nm, whereas the deoxy form peaks at about 557 nm (Appleby, 1992). A Soret band at about 412 nm is characteristic of the optical spectrum for all haemoproteins regardless of their

oxygenation state.

OCCURRENCE OF HAEMOGLOBIN IN THE BIOSPHERE

While the existence of haemoglobin in the animal kingdom has long been common knowledge, the findings of its presence in non animal sources are relatively recent. The evidence accumulated already supports the wide spread occurrence of haemoglobin across the biosphere. Haemoglobins have been found in all major groups of organisms including eubacteria, unicellular eucaryotes, plants and animals (Hardison, 1996).

Two types of haemoglobin are known to exist in vertebrates. Myoglobin exists as a monomer and is found in various muscle tissues where it serves as an oxygen storage and delivery protein. The oxygen carrying erythrocyte haemoglobin is composed of two α and two β subunits that are identical in 50% of their amino acid sequence regardless of the source species. It has been proposed that the two genes descended from one haemoglobin gene in an ancestral jawed vertebrate only about 450 million years ago (Hardison, 1996). These two proteins fold into globin structures that are virtually identical with that of myoglobin. In fact, Perutz (1978) described the red blood cell haemoglobin as "just four myoglobin molecules put together". Apart from vertebrates, haemoglobins have been found in a range of invertebrates from nematodes (Dixon *et al.*, 1992) to annelids and arthropods (Riggs, 1991; Sherman *et al.*, 1992).

Haemoglobins isolated from plants fall into two general categories. Symbiotic haemoglobins are found in plants entering symbiosis with microorganisms. They are best represented by leghaemoglobins that are produced in the nodules of legume plants (Kubo,

1939) where they function in controlling oxygen supply to nitrogen fixing *Rhizobia* (Wittenberg *et al.*, 1974). The nonsymbiotic haemoglobins, also called plant haemoglobins, were first found in dicotyledonous *Trema tomentosa* (Bogusz *et al.*, 1988), but the wide spread existence of haemoglobin in the plant kingdom was truly proven by its discovery in monocotyledonous barley (*Hordeum vulgare*) (Taylor *et al.*, 1994), rice (*Oryza sativa*) (Sasaki *et al.*, 1994) and algae, *Chlamydomonas eugametos* (Couture *et al.*, 1994). Although the differences in the primary sequence between haemoglobins from plant and animal sources are significant (Riggs, 1991), structural analysis of soybean leghaemoglobin suggests that they all fold into virtually identical globin structures (Vanhstein *et al.*, 1975).

Haemoglobins have also been found in far less advanced species representing protozoa (Iwaasa *et al.*, 1990), fungi (Keilin, 1953) and bacteria (Wakabayashi *et al.*, 1986). Fungal haemoglobin, isolated from *Saccharomyces cerevisiae*, has both haem and flavin binding domains (Oshino *et al.*, 1973). Similar two-domain haemoglobins are known in bacterial species of *Alcaligenes eutrophus* (Probst *et al.*, 1979) and *Escherichia coli* (Vasudevan *et al.*, 1991). These proteins are called flavohaemoglobins, since despite having two binding domains they produce the classic globin structure. Interestingly, a one domain haemoglobin that shares 26% identity with lupin leghaemoglobin was isolated from bacterium *Vitreoscilla* (Wakabayashi *et al.*, 1986).

Symbiotic haemoglobins

Nodulating plants are able to participate in free nitrogen fixing symbioses with bacterial micro symbionts. An obvious benefit of such symbioses to plants is the additional source of nitrogen. *Rhizobium* and *Bradyrhizobium* of the family *Rhizobaceae*, and *Frankia* a member of Actinomycetes are the only known micro symbionts (Ishizuka, 1992). The best characterized are the symbioses of *Rhizobium* and *Bradyrhizobium* bacteria with leguminous plants. The only nonleguminous plant known to be nodulated by *Rhizobium* or related members of *Rhizobaceae* is *Parasponia*, a member of *Ulmaceae* (Appleby, 1992). *Frankia* nodulates a variety of nonleguminous dicotyledonous plants forming actinorhizal symbioses (Silvester and Winship, 1990). Reduction of free nitrogen in all of these symbiotic systems depends on the activity of bacteria encoded nitrogenase. The nitrogenase reaction, described as:

$N_2 + 8H^+ + 8Fd^- + 16MgATP^{2-} + 18H_2O \rightarrow 2NH_4^+ + 2OH^- + H_2 + 8Fd + 16MgADP^- + 16H_2PO4^-$ (Fd = ferredoxin)

requires substantial amounts of energy in the form of ATP (Kennedy and Tchan, 1992). These energy needs have to be met by bacteroid respiration. Paradoxically, the oxygen that is necessary for respiration readily inhibits the activity of nitrogenase.

It has been well documented that haemoglobin found in symbiotic nodules plays a crucial role in the control of oxygen supply within nodules. It allows the system to meet the oxygen requirement for bacteroid respiration while keeping oxygen tensions low enough to sustain the function of nitrogenase (Appleby, 1984). Other than haemoglobin,

factors such as mechanical barriers have also been implemented in the control of nodule oxygen supply (Hunt and Layzell, 1993).

The apparent absence of these haemoglobins in parts of the plant other than nodules, led some researchers to believe that haemoglobins were produced by bacteroids. Further research, however, has proven that they are in fact encoded and produced by plants. The first evidence came when Lb apoprotein was synthesized in a wheat germ system with mRNA isolated from nodules as templates (Verma *et al.*, 1974). Later it was shown that leghaemoglobin cDNA, derived from soybean nodule mRNA, hybridized to the soybean genomic DNA but not to DNA isolated from *Rhizobium* (Sidloi Lumbroso *et al.*, 1978). Numerous isoforms of leghaemoglobin are found in legume plants. The four major isoforms isolated from soybean, Lba, Lbc1, Lbc2 and Lbc3, represent products of separate genes (Fuchsman and Appleby, 1979), while the four minor forms, Lbb, Lbd1, Lbd2 and Lbd3 result from post-translational acetylation of the major forms (Appleby, 1992).

Other than legumes, symbiotic haemoglobin sequences have been found in the DNA of actinorhizal plants (Roberts *et al.*, 1985). The first such haemoglobin was isolated from actinorhizal nodules of *Casuarina glauca* (Fleming *et al.*, 1987). Haemoglobin in these nodules remained firmly bound to cell membranes. The other interesting property of this protein was that it has a lower oxygen affinity than leghaemoglobins, suggesting that the *Frankia* endophyte of *Casuarina* might be more oxygen-tolerant than *Rhizobium* (Fleming *et al.*, 1987). Subsequently, *Casuarina* haemoglobin gene was cloned and characterized (Christensen *et al.*, 1991). The presence of a high concentration of a

haemoglobin-like protein in nodules of *Casuarina cunninghamiana* and *Myrica gale* was indicated by the 416 to 420 nm absorption band of carboxyhaemoglobin (Tjepkema and Asa, 1987). The protein from *Myrica gale* was later purified and confirmed to be a true haemoglobin (Pathirana and Tjepkema, 1995).

Isolation of haemoglobin from *Parasponia anderssoni*, the host of *Rhizobium* endophyte (Appleby *et al.*, 1983), attracted great attention among researchers. Not only was it the first haemoglobin in the plant kingdom to be found in other than leguminous plant source, but also it was expressed in both nodules and nonsymbiotic parts of the plant. This implicated a symbiotic as well as nonsymbiotic function for this protein and led to the hypothesis that the presence of haemoglobin may extend beyond nodulating plants.

Nonsymbiotic haemoglobins

The *Parasponia* haemoglobin cDNA was used as a probe to detect a haemoglobin gene in *Trema tomentosa* (Bogusz *et al.*, 1988). Although *Trema* belongs to the same family as *Parasponia* family (*Ulmaceae*) it has no known symbiotic association. This finding made it apparent that plant haemoglobins exist more widely than previously thought and may have other than symbiotic functions.

The search for other nonsymbiotic plant haemoglobins that followed the *Trema* Hb discovery, was for many years unsuccessful, due to poor cross-hybridization between symbiotic Hb cDNA probes and nucleic acids isolated from nonsymbiotic plants. The breakthrough came with the serendipitous discovery of haemoglobin in barley (Taylor *et al.*, 1994). Using the barley Hb cDNA probe the same authors detected related sequences

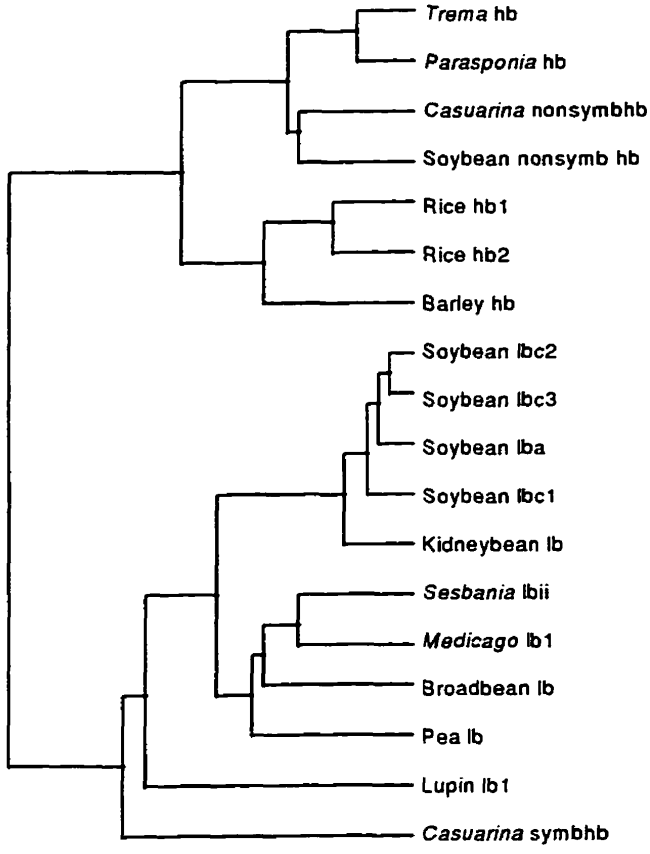
in maize, wheat, rye and triticale (Taylor *et al.*, 1994). This work made it clear that haemoglobins are indeed wide spread in plants. The same year brought reports of the presence of two non-symbiotic haemoglobins in rice (Sasaki *et al.*, 1994) and in chloroplasts of the algae, *Chlamydomonas eugametos* (Couture *et al.*, 1994). To date nonsymbiotic haemoglobins have been reported in a variety of plants, including the symbiotic soybean (Andersson *et al.*, 1996) and actinorhizal *Casuarina* (Christensen *et al.*, 1991; Jacobsen-Lyon *et al.*, 1995).

The plant haemoglobin protein similarity tree (Fig.1) (Andersson *et al.*, 1996), shows clearly that symbiotic and nonsymbiotic haemoglobins, regardless of the source species, represent two distinct and coherent subdivisions within the haemoglobin family. Analysing oxygen binding characteristics of plant haemoglobins, one can observe another distinction between these two haemoglobin types. The nonsymbiotic proteins (Duff *et al.*, 1997; Arredondo-Peter *et al.*, 1997) are much more oxygen avid than their symbiotic counterparts (Gibson *et al.*, 1989).

A new insight into the nonsymbiotic haemoglobins have been recently brought by the isolation of two nonsymbiotic Hb genes and their products in *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). AHb1 gene represents, in sequence and oxygen affinity, a classical nonsymbiotic haemoglobin, whereas AHb2 shows higher similarity in both sequence and oxygen binding to the symbiotic Hbs. The subsequential identification of a AHb2 homologue from *Brassica napus* (unpublished) has led the authors to propose a further division within the nonsymbiotic Hbs. They suggest that classical nonsymbiotic Hbs be called "class 1" nonsymbiotic Hbs and the AHB2 like genes "class 2" nonsymbiotic

Hbs. The two nonsymbiotic haemoglobin genes isolated from rice (Sasaki *et al.*, 1994) were not included in the comparison of the class 1 and class 2 genes. It would be valuable, from the evolutionary and functional point of view, to know whether the class 2 haemoglobins extend into monocotyledonous plants.

Figure.1 Plant haemoglobin protein similarity tree (from Andersson et al., 1996).



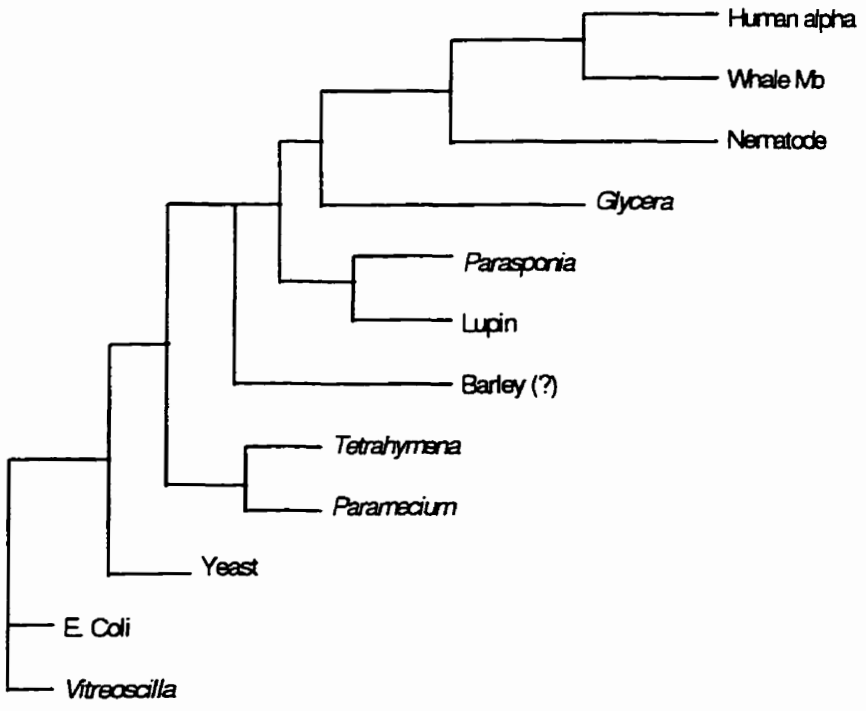
Despite numerous reports on the identification of nonsymbiotic haemoglobins and their sequence analysis for evolutionary studies, little is known about the function of these proteins in plants.

ORIGIN AND EVOLUTION OF PLANT HAEMOGLOBINS

Since the discovery of leghaemoglobins almost sixty years ago (Kubo, 1939), questions have been raised regarding the origin and evolution of haemoglobin. For almost fifty years the apparent lack of haemoglobin presence in other than legume plant sources, supported a notion that haemoglobins have been introduced to plants by horizontal transfer from the animal kingdom, possibly through an insect or nematode vector (Appleby *et al.*, 1984). Arguments against this hypothesis came with relatively recent identification of haemoglobins in nonlegume and nonsymbiotic plant sources as well as findings of Hbs in protozoa, fungi and bacteria.

It is now widely accepted that plant and animal haemoglobins originate from the same ancestral globin gene, about 1500 million years ago, and have been shaped by vertical evolution. Moreover, finding haemoglobins in bacteria shows that haemoglobin is a truly ancient gene, preceding the divergence of procaryotes and eucaryotes, about 1800 million years ago (Appleby, 1992; Hardison, 1996). Zhu and Riggs (1992) analysed the phylogenesis of haemoglobins and proposed the phylogenetic tree shown in Figure 2.

Fig. 2 Phylogenetic analysis of the relationship between various haemoglobins (from Zhu and Riggs, 1992; Barley Hb was not included in the original tree)



Gene structure analyses are usually very helpful in tracking the steps of a gene's evolution. Bacterial haemoglobin, just like all bacterial genes, does not have any introns. Nor does the fungal haemoglobin gene (Zhu and Riggs, 1992). Symbiotic plant haemoglobins (Jensen *et al.*, 1981) as well as the nonsymbiotic plant Hb genes have a three intron and four exon structure (Hardison, 1996). The nonsymbiotic Hb gene structure was demonstrated recently by Guy, Sowa and Hill (1997), who showed that barley Hb gene possesses three introns in exactly identical positions to those found in soybean and *Parasponia*. Interestingly, animal haemoglobin genes have either three

(nematode), two (most arthropods, vertebrate and annelid) or no (insect) introns (Hardison, 1996). Close analysis shows that the two introns of the vertebrate, arthropod and annelid Hb genes are in the same positions as the first and third introns of the plant genes. It would appear that these animal genes lost the middle intron during the course of evolution. The third, middle intron found in the nematodes *Ascaris* and *Pseudoterranova* (Dixon *et al.*, 1992; Goldberg, 1995) might represent the link between plant and animal genes. Moreover, the high oxygen affinity of nematode Hb (Goldberg, 1995) is within the range of nonsymbiotic plant haemoglobins. The placement of this middle intron in nematode genes is close, but not identical to its position in plant Hb genes. This may suggest either an intron sliding or independent insertion. Interestingly, in *Chlamydomonas* the middle intron is in the same position as in plant Hbs, however the two remaining introns come in different positions than in plant and animal genes (Couture *et al.*, 1994). These phenomena of sliding or independently inserted introns may be difficult to explain, but the overwhelming evidence supports vertical, and at least 1800 million years long, evolution of haemoglobin genes (Hardison, 1996).

The very existence of two types of haemoglobins in plants raises a question: which was first? Symbiotic Hb genes are restricted only to plants capable of participation in N_2 fixing symbioses, whereas the nonsymbiotic genes are far more widespread. The finding of nonsymbiotic haemoglobins in symbiotic plants (Andersson *et al.*, 1996; Christensen *et al.*, 1991) favour the hypothesis that symbiotic haemoglobins arose through duplication of nonsymbiotic genes followed by speciation to fit the needs of symbiosis. Further evidence comes from the identification of noduline motifs 5'-AAAGAT-3' and 5'-CTCTT-3' in

symbiotic genes of legumes and actinorhizal plants (Ramlov *et al.*, 1993; Christensen *et al.*, 1991) These motifs are absent from the nonsymbiotic haemoglobin gene of *Casuarina* (Jacobsen-Lyon *et al.*, 1995), which may suggest that symbiotic haemoglobins evolved from the nonsymbiotic ones in response to the needs of symbiosis. In *Parasponia* one haemoglobin gene, whose sequence shows more homology with the nonsymbiotic than with symbiotic genes, has both symbiotic and nonsymbiotic functions (Landsmann *et al.*, 1986; Bogusz *et al.*, 1990). Although the *Parasponia* situation supports the idea of a nonsymbiotic gene duplication leading to the symbiotic genes, an alternative explanation may be suggested by the most recent finding of class 2 nonsymbiotic genes in *Arabidopsis* and *Brassica* (Trevaskis *et al.*, 1997). The class 2 nonsymbiotic haemoglobin shares more similarities with symbiotic than with nonsymbiotic genes and could be used to argue that the symbiotic haemoglobin genes gave rise to nonsymbiotic ones. Nevertheless, the stronger evidence is in support of the earlier hypothesis and it is generally accepted that the nonsymbiotic haemoglobins were evolutionary predecessors of the more specialized symbiotic Hbs (Trevaskis *et al.*, 1997).

Since haemoglobins are not the only haem binding proteins, valid questions have been raised concerning the possible common origin of haemoglobins and other haemoproteins (Hardison, 1996). It has been proposed, based on similarities between amino acid sequences, that globins evolved from cytochrome b_5 (Hardison, 1996; Runnegar, 1984).

Little is known about the functional evolution of haemoglobin. Hardison (1996) hypothesizes that metal-bound porphyrin rings were present when photosynthesis evolved.

Such a ring chelating Mg^{2+} is a basic structure of chlorophyll. Haemoproteins could have been used at that time to protect the cell from oxygen produced by photosynthetic processes. From there, evolution would take them to electron transfer agents, such as cytochromes, and oxygen transport proteins. Today haemoglobins have various functions in different organisms, from accepting electrons in bacteria through buffering oxygen tensions in muscles of vertebrates and plant nodules to transporting oxygen in animals.

TOWARDS THE FUNCTION OF NONSYMBIOTIC HAEMOGLOBIN IN PLANTS

Some useful information for delineating a function of a protein might be gained from studying the patterns of its expression and regulation of this process, as well as analysing the chemical properties of the molecule. The known functions of related proteins will also provide valid clues. The aspiration of this thesis is to add to the understanding of the function of nonsymbiotic haemoglobins in plants, therefore this part of the review will discuss expression of nonsymbiotic haemoglobins, and present some of the properties and functions of known haemoglobins.

Haemoglobin gene expression and regulation

The expression of haemoglobin genes in plants is highly tissue-specific. Symbiotic haemoglobins are expressed only in nodule tissue (Appleby, 1992). Noduline motifs present in promoters of leghaemoglobins and many other noduline genes direct their expression specifically to the nodules. The 5'-AAAGAT-3' and 5'-CTCTT-3' *cis*-elements, known as noduline motifs, are separated in leghaemoglobin only by 6 to 7 nucleotides. It

appears that the latter motif is of greater importance for the expression of nodule specific genes (Ramlov *et al.*, 1993; Stougaard *et al.*, 1987; Szczyglowski *et al.*, 1994). The same element is found in promoters of symbiotic haemoglobins from the actinorhizal plant *Casuarina* (Jacobsen-Lyon *et al.*, 1995). In place of the first motif , 5'-AAAGAT-3', *Casuarina* symbiotic promoter contains a slightly different 5'-CAAGAT-3' sequence. Functioning as oxygen buffers symbiotic haemoglobins are expressed at a very high level . Leghaemoglobin in soybean nodules is found at concentrations of 700 μM (Gibson *et al.*, 1989).

The nonsymbiotic haemoglobins, or their respective mRNAs, are found in metabolically active tissues such as roots (Taylor *et al.*, 1994; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997; Andersson *et al.*, 1996), aleurone (Taylor *et al.*, 1994) and vascular tissues of leaves stems and seedling cotyledons (Andersson *et al.*, 1996). The expression levels of these proteins are relatively low in the order of 20-30μM (Duff *et al.*, 1997).

There is no published information on the promoter sequences of haemoglobins from monocotyledonous sources, although the attempts to isolate haemoglobin promoter from barley have been undertaken quite a while ago. The sequences of nonsymbiotic haemoglobins from *Casuarina*, *Parasponia*, *Trema* and soybean show no noduline motifs at the spacing found in symbiotic genes (Andersson *et al.*, 1997; Jacobsen-Lyon *et al.*, 1995). Instead , similar but not identical sequences were found in these promoters. An examination of *Casuarina* nonsymbiotic haemoglobin promoter, by deletion studies in transgenic *Lotus corniculatus*, revealed that these sequences have no impact on the gene

expression and that the critical elements were located further upstream (Jacobsen-Lyon *et al.*, 1995).

Casuarina nonsymbiotic haemoglobin is strongly expressed in root, leaf and stem tissues and, at barely detectable levels, in nodules. This pattern is maintained in the transgenic legume, *Lotus corniculatus* (Jacobsen-Lyon *et al.*, 1995). Similarly, promoters of *Trema* and the bifunctional *Parasponia* Hb genes, direct expression of the GUS reporter gene in roots and nodules of transformed *L. corniculatus*. However in these cases expression in nodules appears to be stronger than in roots (Andersson *et al.*, 1997; Bogusz *et al.*, 1990). In all cases, nonsymbiotic genes were not expressed in *Rhizobium* infected cells (Andersson *et al.*, 1997; Jacobsen-Lyon *et al.*, 1995).

Haemoglobins in barley (Taylor *et al.*, 1994), maize (Silva, 1997), and *Arabidopsis* (Trevaskis *et al.*, 1997) are stress inducible, suggesting that at least some nonsymbiotic haemoglobins are involved in stress response in plants. Low oxygen tensions induce expression of barley, maize and *Arabidopsis* AHB1 (class 1) Hb genes. Barley haemoglobin is induced at atmospheric oxygen levels of about 5 percent. The induction is rapid, with increased transcription becoming evident within 30 min and maximum expression occurring about 12 hours after placing the tissue under a nitrogen atmosphere (Taylor *et al.*, 1994). AHB1, in addition, is also induced by 1% sucrose. AHB2 (class 2) gene of *Arabidopsis* is induced by chilling (Trevaskis *et al.*, 1997).

Nie and Hill (1997) studied signal transduction leading to the induction of Hb expression in barley aleurone tissue. Their work shows that blocking of mitochondrial electron transport chain, as well as blocking of ATP synthase activity, in the presence of

oxygen, results in haemoglobin induction at the levels similar to those observed under oxygen stress. An obvious conclusion, drawn by these authors, is that Hb induction is triggered not by the lack of oxygen *per se*, but by the resulting drop in ATP synthesis.

Properties of nonsymbiotic haemoglobins

Recent isolations of nonsymbiotic haemoglobins from barley, rice, soybean and *Arabidopsis* enabled studies of the chemical properties of these proteins. The first to be characterized was haemoglobin from barley (Duff *et al.*, 1997), which was followed by less detailed studies on haemoglobins from rice (Arredondo-Peter *et al.*, 1997) and *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). In all cases recombinant proteins were produced in bacteria, then purified and used to determine subunit structure, spectral characteristics and, most importantly, ligand binding kinetics. In the case of barley haemoglobin the results obtained with recombinant protein were confirmed with the native one (Duff *et al.*, 1997). Barley haemoglobin is a homodimer with a subunit molecular mass of 18.5 kDa. Each subunit possesses a haem chromophore giving spectral characteristics of a Soret band at 412 nm with visible bands at 540 and 576 nm. The optical spectra of barley haemoglobin, in both ferrous and ferric state, are reminiscent of many low spin 6-C haemoproteins (Duff *et al.*, 1997). These include ferrous haemochromogen, ferrous protohaem cytochromes including cytochrome *b*, ferrous cytochrome *c*, ferrous myoglobin or horseradish peroxidase cyanides and ferrous myoglobin or leghaemoglobin nicotinates .

A characteristic of all studied nonsymbiotic haemoglobins is a very slow ligand dissociation reaction, resulting in extremely high ligand affinities of these proteins

(Table 1). The exceptions are the bifunctional haemoglobin from *Parasponia* and the class 2 haemoglobin from *Arabidopsis*. Both of these proteins function either in symbiotic associations or share extensive sequence homology with symbiotic Hbs. These ligand binding kinetics, apart from sequence differences, make a clear functional division between the symbiotic and nonsymbiotic plant haemoglobins.

Functions of haemoglobins

The tetrameric vertebrate haemoglobins from erythrocytes have been long known to transport oxygen from lungs, gills or skin of some animals to the tissue where it is utilized as the terminal electron acceptor in catabolism (Giardina *et al.*, 1995). Besides this basic function of oxygen transport, other functions have also been proposed. For example, it has been suggested that human haemoglobin functions as a molecular heat transducer through its oxygenation-deoxygenation cycle, and as a modulator of erythrocyte metabolism (Giardina *et al.*, 1995). It has also been proposed that haemoglobin may play a role in erythrocyte senescence and malaria resistance (for references, see Giardina *et al.*, 1995).

Unlike vertebrate erythrocyte haemoglobin, cytoplasmic haemoglobins, including myoglobin and plant haemoglobins, do not exhibit the sigmoid oxygenation curve due to the absence of haem-haem interaction. The functions of plant haemoglobins and myoglobin, thus, should differ from that of vertebrate erythrocyte haemoglobin. It has been proposed that the cytoplasmic haemoglobin may facilitate oxygen diffusion (reviewed by Wittenberg and Wittenberg, 1990), or function as a mediator of oxidative

phosphorylation, or even as a terminal oxidase or an oxygen sensor (Appleby *et al.*, 1988). The next part of this review, will focus on the functions of some cytoplasmic haemoglobins.

Haemoglobin as an oxygen diffusion facilitator and in oxygen storage

In order to act as an oxygen carrier, or to facilitate oxygen diffusion within the cell, haemoglobin must fulfill two ground conditions. First, it must be present in sufficient quantities to make it effective in delivering oxygen in quantities that maintain oxygen demands of the cell. To be effective in facilitating diffusion of oxygen, its concentration must be high enough to create a gradient that supplies oxygen faster than the rate of diffusion of free oxygen. Second, it has to exist in a state of partial oxygenation, that is, a certain percentage of haemoglobin molecules must be in an oxygenated state and the equilibrium between the oxygenated and deoxygenated haemoglobin ought to be maintained (Appleby, 1992). In fact, some haemoglobins, including myoglobin, are found to function in partial oxygenation state in various tissues (Wittenberg and Wittenberg, 1990). In myoglobin dependent systems, such as vertebrate heart and muscle, free oxygen diffusion within the cell is extremely slow due to the low oxygen solubility in thick cytoplasm. Free oxygen pressure in mitochondria is at very low levels, about 20 to 25 fold lower than in erythrocyte. This creates a gradient of oxygen pressure, in which the diffusion of oxygen bound haemoglobin generates a flux of oxygen. It has been estimated that the concentration of myoglobin in the heart muscle exceeds free oxygen concentration by over thirty times (Wittenberg and Wittenberg, 1990), therefore the diffusion of

myoglobin bound oxygen is greater than of free O_2. It has been well documented that leghaemoglobin plays a key role in regulation of oxygen supply to the respiring bacteroids. A membrane separates the bacteroid from the host plant cell cytoplasm where the concentration of leghaemoglobin may reach nearly 4 mM (Appleby, 1984). With the estimated 20% oxygenation of leghaemoglobin, the steady state concentration of free dissolved oxygen at the bacteroid surface would be in the neighbourhood of 10 nM (Appleby, 1984). The leghaemoglobin bound oxygen is, then, at a concentration of about 0.75 mM. That is 75, 000 times greater than that of free oxygen, indicating that the entire flux of oxygen to the bacteroid is leghaemoglobin mediated (Appleby, 1992).

Another significant factor, in haemoglobin facilitated oxygen transport or diffusion, are the kinetics of oxygen binding. At a given concentration of free, dissolved oxygen, haemoglobin should be able to efficiently bind O_2 and release it at a non limiting rate. Both leghaemoglobin and vertebrate myoglobin have the oxygen binding kinetics that make them fit to perform their respective functions (Table 1). Although the oxygen affinity (dissociation constant, K_D), calculated as the 'off' rate (k_{off}) divided by the 'on' rate (k_{on}), of symbiotic haemoglobins can be ten fold higher than that of myoglobin (Table 1), the oxygen dissociation reactions are almost equally fast (Appleby, 1992). This makes them a model for facilitated oxygen diffusion. The oxygen 'on' rate constants of $165\mu M^{-1}$ sec^{-1} of *Casuarina* symbiotic Hb and 120 μM^{-1} sec^{-1} of soybean leghaemoglobin (Table 1) are almost as high as the theoretical diffusion-limited rate for molecules of respective molecular sizes of oxygen and haemoglobin (Appleby, 1992). This makes leghaemoglobin extremely effective in getting hold of all available oxygen.

Oxygen storage by myoglobin in aquatic animals, such as whales, seals and crocodiles is significant, which enables them to submerge for extended periods of time. The myoglobin concentration in these animals is about ten times greater than in terrestrial mammals (Voet and Voet, 1990). When they are on the surface, large amounts of oxygenated myoglobin are formed, which later serve as an oxygen source during diving when oxygen levels in erythrocytes decline.

Haemoglobins as oxygen sensors

Changing conditions in a natural environment may often be life threatening to living organisms. Environmental stresses such as cold, heat, drought and flooding are especially dangerous to plants since they can not simply run away from them. Several defence mechanisms, often involving metabolic alterations, have evolved to increase stress survival chances. The first step in signal transduction pathways that activate these mechanisms must be the detection of the changing conditions. During inadequate oxygen supply, anaerobic metabolism is often turned on as an alternative pathway of energy production. A molecule that senses oxygen levels has been proposed in many species from bacteria to mammals (Acker, 1994). Haemoprotein would be a natural candidate for an oxygen sensor, and in fact there is substantial evidence to support haemoprotein based oxygen sensing (Gilles Gonzalez *et al.*, 1994; Poole *et al.*, 1994a; Acker, 1994; Goldberg *et al.*, 1988).

In mammals, erythropoietin, a hormone, is involved in the regulation of haemoglobin synthesis (Minegishi *et al.*, 1994). The synthesis of erythropoietin is, in turn,

stimulated under hypoxia (Goldberg *et al.*, 1987; Goldberg *et al.*, 1988). This hypoxic induction of erythropoietin gene, in human hepatoma cells, is blocked by inhibitors of haem synthesis and by carbon monoxide, which locks haemoproteins in their oxy-conformation, indicating that haemoprotein is involved in oxygen sensing (Goldberg *et al.*, 1988).

In bacteria, two proteins, Fix L (Gilles Gonzalez *et al.*, 1991; Gilles Gonzalez *et al.*, 1994) and Hmp (Poole *et al.*, 1994a) have been proposed as oxygen sensors. Both have a haem domain but, in addition, FixL possesses a protein kinase domain (Monson *et al.*, 1992) while Hmp has a flavoprotein domain with NADH oxidase activity (Andrews *et al.*, 1992; Cooper *et al.*, 1994). In both cases the occupancy of haem by oxygen affects the activity of the second domain. In oxygenated Hmp flavin is predominantly oxidized, whereas in the absence of oxygen flavin is reduced allowing Hmp to act as a reductase. FixL, found in *Rhizobium meliloti*, is membrane-bound. Upon deoxygenation, its kinase domain is activated, phosphorylating a transcriptional factor that activates a cascade reaction leading to the expression of critical nitrogen fixation genes (Gilles Gonzalez *et al.*, 1991; Gilles Gonzales *et al.*, 1994).

It has been proposed (Appleby *et al.*, 1988), that the nonsymbiotic plant haemoglobin may act as an oxygen sensor. A conformation change, in deoxygenated haemoglobin, would trigger the activation of anaerobic metabolism. The low level of expression of nonsymbiotic haemoglobins may not support the oxygen transport or storage, but the concentration present would be enough to support the sensing function.

Haemoglobin as a terminal oxidase

The presence of a haem domain in cytochromes as well as the possible common origin of cytochromes and globins invites a theory of possible functional similarity between the two groups. Haemoglobins of yeast and bacteria have been proposed to function as terminal oxidases, the sites of oxygen reduction (Wittenberg and Wittenberg, 1990). Both yeast and *E. coli* haemoglobins have two prosthetic groups, haem and a flavin (Zhu and Riggs, 1992). The structure of yeast haemoglobin resembles that of flavocytochrome b_2 (Xia *et al.*, 1987). The flavin domain of yeast haemoglobin is homologous with members of a flavoprotein family that includes ferredoxin reductase, nitric oxide synthase, and cytochrome P-450 reductase (Zhu and Riggs, 1992). Binding of oxygen by the haem domain may be accompanied by conformation change which acts as a switching mechanism to control the activity of the second domain. Destruction of yeast haemoglobin in the living cell did not inhibit culture growth or oxygen uptake (Oshino *et al.*, 1973). The same is, however, true for terminal oxidases of many bacteria, where alternative pathways are available (Wittenberg and Wittenberg, 1990).

Vitreoscilla is an obligate aerobic bacterium that often lives in oxygen poor environments. Its haemoglobin is a dimer of identical subunits that demonstrate a significant amino acid sequence similarity with eukaryotic globins (Wakabayashi *et al.*, 1986). Expression of *Vitreoscilla* Hb gene increases greatly under limiting oxygen pressure. Intracellular expression of *Vitreoscilla* haemoglobin (VHb) in *E. coli* significantly improves ATP production and culture growth under oxygen limited conditions (Khosla and Bailey, 1988; Kallio *et al.*, 1994) by maintaining energy efficient,

aerobic respiration. When expressed in baker's yeast, VHb alters the aerobic metabolism of the yeast, probably by affecting the mitochondrial electron transport pathway (Chen *et al.*, 1994). Moreover, expression of VHb in *E. Coli* that lack terminal oxidases, cytochrome *o* and *d*, restores culture growth in the presence of aerobic substrates, such as succinate and lactate, and restores aerobic respiration (Dikshit *et al.*, 1992).
The same haemoglobin expressed in tobacco resulted in increased growth rates and seed germination, however the mechanism of this effect has not yet been identified (Holmberg *et al.*, 1997).

Myoglobin-mediated oxidative phosphorylation

Myoglobin-mediated oxygen delivery has been well established in cardiac myocytes (Wittenberg and Wittenberg, 1987). Recently a new function for myoglobin has been proposed (Wittenberg and Wittenberg, 1990). Carbon monoxide, through a tight binding to the haem, blocks the ability of myoglobin to combine with oxygen. This inhibition of myoglobin oxygen binding, has been shown to abolish about a third of oxygen uptake and a significant part of ATP synthesis in resting cardiac myocytes under experimentally imposed conditions of superabundant oxygen. Oligomycin, a specific inhibitor of mitochondrial ATP synthase, blocks mitochondrial ATP synthesis. In the presence of oligomycin, carbon monoxide is no longer effective in blocking of oxygen uptake by the myocytes. These effects tie myoglobin directly to the mitochondrial oxidative phosphorylation and is termed myoglobin-mediated oxidative phosphorylation (Wittenberg and Wittenberg, 1990). Furthermore, rotenone blockade of electron transport

from NADH to ubiquinone, inhibits myoglobin-mediated oxidative phosphorylation, which proves that the phenomenon depends on electron transport in mitochondria. In this system something other than oxygen must travel across the mitochondrial membrane to mediate the effects of myoglobin on oxidative phosphorylation, since oxygen uptake is not affected by oligomycin. To explain the phenomenon of myoglobin mediated oxidative phosphorylation Wittenberg and Wittenberg (1990) propose a model, in which reducing equivalents for reduction of myoglobin-bound oxygen come from a transmembrane protein capable of vectorial separation of electron charges or protons. In bacteria it is easy to imagine a complex between a transmembrane protein in the plasmalemma and cytosolic haemoglobin. In eucaryotes, because of the outer mitochondrial membrane separation of the electron transport chain and cytosol, an outer membrane and possibly intermembrane space protein would have to couple the inner membrane-spanning protein and myoglobin.

Other functions

A hydrogen sulfide-reactive haemoglobin has been found in symbiont- harbouring gills of the clam *Luccina pectinata* and other sulfide-interface clams (Wittenberg and Wittenberg, 1990). This haemoglobin exists as a mixture of ferric and ferrous forms. In the presence of hydrogen sulfide the ferric form binds sulfide with an extraordinarily high affinity, K_D = 3.4 nM. This shifts equilibrium between ferric and ferrous haemoglobin resulting in a quick conversion of all haemoglobin into ferric sulfide form. A hypothesis suggests that this haemoglobin functions in augmenting the hydrogen sulfide flow to the symbiont.

Symbiotic haemoglobin in plant root nodules and in symbiont-harbouring mollusc gills have been implicated as iron chelators in peribacteroid space. More work is needed to elucidate and explain these possible functions of haemoglobins.

LIMITING OXYGEN STRESS IN PLANTS

Since the nonsymbiotic haemoglobins of barley (Taylor *et al.*, 1994) and *Arabidopsis* (Trevaskis *et al.*, 1997) have been shown to be hypoxia inducible, a possibility exists that at least some nonsymbiotic Hbs may be a part of anaerobic response in plants. This part of the review will concentrate on the responses of plants to anaerobiosis and the anaerobic metabolism.

Most terrestrial higher plants, with a few exceptions such as rice and *Echinochloa* (Rumpho and Kennedy, 1981), absolutely depend on oxygen for energy production in amounts sufficient for growth and development. The supply of oxygen to a tissue depends on its concentration and its diffusion rate in the surrounding medium. In natural environments periodic water logging of the soil may occur, even with certain regularity. Since the diffusion of oxygen is about 10,000-fold slower in water than in air (Armstrong, 1979), respiration of plant roots and soil microorganisms leads to a quick depletion of oxygen, resulting in hypoxic conditions. A consequence of this are chemical and biological changes of soil properties that adversely affect plant growth. Apart from the obvious lack of sufficient oxygen amounts, these changes include reduction of soil redox potential, changes of the soil pH and increased levels of CO_2 and ethylene (Atwell *et al.*, 1985).

Two general strategies have evolved to overcome the effects of oxygen

unavailability. First is a biochemical and physiological fast response by altering metabolism, growth rate and nutrient distribution (Barclay and Crawford, 1982). Second are long term morphological adaptations creating alternative routes of oxygen supply from the unaffected parts, thus sustaining aerobic respiration and oxidative phosphorylation. Different plant species and even different tissues, nevertheless, display varying levels of low oxygen stress tolerance. Depending on the species, the tolerable time length of anoxia ranges from a few hours to several days (Crawford and Braendle, 1996). Similarly, the seeds of some plants can germinate in anoxia while others require full oxygen availability.

The most studied, and characteristic not only to plants but to most organisms, is fermentative, mostly ethanolic or lactic metabolism, with glycolysis as the main source of ATP synthesis.

Anaerobic metabolism

Mitochondrial respiration depends on oxygen as the terminal electron acceptor in the mitochondrial electron transport process. Terminal oxidases in plant tissues have a rather high K_m (about 100 nM) for oxygen (Appleby, 1992). Lack of sufficient oxygen concentrations, therefore, stops the mitochondrial electron transport chain and oxidative phosphorylation. The tricarboxylic acids cycle (TCA) becomes inoperative due to accumulation of reduced pyridine nucleotides, NADH and $FADH_2$, that can not be reoxidised without an electron acceptor. Under such conditions, the energy production moves to the cytoplasm, where glycolysis becomes the only pathway of ATP synthesis. Since glycolysis alone is 19 (theoretically calculated) times less effective in ATP

production than when coupled with mitochondrial respiration, a substantial increase in glycolytic activity is, therefore, necessary if a cell or tissue is to survive the conditions of insufficient oxygen availability.

The rate of glycolysis greatly depends on the availability of oxidizing factor, NAD. The accumulation of NADH, reduced both in glycolysis and the TCA cycle, results in a switch to a fermentative pathway, whose purpose is reoxidation of NADH in order to maintain continuous ATP production through substrate phosphorylation in glycolysis.

Although several pathways have been proposed to reoxidize NADH in the absence of oxygen (Ricard *et al.*, 1994; Kennedy *et al.*, 1992), fermentative pathways leading to ethanol, lactate and alanine production are believed to be the major ones used to recycle pyridine nucleotides during anaerobiosis in plants (Ricard *et al.*, 1994). In all cases, pyruvate, the end product of glycolysis, is converted to fermentative end products with the concomitant oxidation of NADH.

In higher plants ethanolic fermentation is overall the most active fermentative process. Pyruvate is converted to acetaldehyde in a reaction catalysed by pyruvate decarboxylase (PDC), which is followed by alcohol dehydrogenase (ADH)-catalysed conversion of acetaldehyde to ethanol. This ADH catalysed reduction of acetaldehyde uses NADH and is the source of NAD for glycolysis. The PDC reaction releases carbon dioxide, therefore high CO_2 release rates are often observed in plants under anoxia.
In lactic fermentation, pyruvate is converted to lactate by the action of lactate dehydrogenase (LDH), a reaction that requires NADH. When alanine is the product of fermentation, the carbon skeleton of alanine is derived from pyruvate while the nitrogen

may come from diverse sources such as amides of asparagine and glutamine and results from protein degradation during anaerobiosis (Menegus *et al.*, 1989).

The sequence of the metabolic events in plants under anaerobiosis is usually such that lactic fermentation is induced first within the first minutes after the onset of hypoxia or anoxia (Roberts *et al.*, 1984). Due to the production of lactate in high amounts, the pH of the cytoplasm decreases rapidly. It has been proposed that the acidification of the cytoplasm by lactate triggers the induction of pyruvate decarboxylase, thus shifting fermentative activity towards the production of ethanol (Ricard *et al.*, 1994). Despite the wide support that this theory received from various studies of anaerobic responses in different plant species, some exceptions have been noted. For example, production of lactate remains at low levels in rice seedlings (Rivoal *et al.*, 1991) and in hypoxia-acclimated maize tips (Xia and Saglio, 1992), where ethanol production commences immediately with the onset of hypoxia. In maize root tips, subjected to anoxia, the time pattern of cytoplasmic pH changes did not follow that of lactic acid accumulation, suggesting that another process is responsible for cytoplasmic acidification (Saint Ges *et al.*, 1991).

Fermentation is not the only metabolic activity in plants under hypoxia. The observation that succinate and malate may accumulate under anaerobic conditions (Vanlerberghe *et al.*, 1990; Vanlerberghe *et al.*, 1989; Davies, 1980; Ricard *et al.*, 1994) suggests a partial operation of the TCA cycle, probably in the reductive direction. A proposed explanation involves carboxylation of phosphoenolpyruvate (PEP) via PEP carboxylase (PEP-C) or PEP carboxykinase (PEP-CK) to produce oxaloacetate, that is in

turn reduced to malate then fumarate and finally succinate (Vanlerberghe *et al.*, 1990; Davies, 1980). Reduction of oxaloacetate to succinate would generate NAD, that could be used to sustain high glycolytic activity. In *Echinochloa pollypogon* all enzymes of the TCA cycle have been found active during anoxia. It has been proposed that in this species the TCA cycle is active in anoxic mitochondria and generates ATP through substrate level phosphorylation (Fox *et al.*, 1994). The authors propose lipid synthesis and possible electron transport to an alternative acceptor as a mechanism of NAD regeneration to sustain TCA substrate level phosphorylation.

Contrary to basic fermentations that are observed in most species, these additional activities may differ significantly between species. For example, the level of malate in some cases does not increase but slowly decreases under anoxia (Vanlerberghe *et al.*, 1990). Succinate accumulation is quantitatively minor compared to lactate or ethanol production. In maize roots, succinate represents only 2.5% of the amount of lactate after 8 h of anoxia (Fan *et al.*, 1988). In rice seedlings under anoxia, the rate of accumulation of succinate is less than 1% of that of ethanol (Rivoal *et al.*, 1989).

Apart from oxygen as the final electron acceptor, other acceptors can enable anaerobic respiration. Nitrate serves as an electron acceptor during respiration in some bacteria (Hochachka and Somero, 1984). The free energy change during complete oxidation of glucose with oxygen or with nitrate as the final electron acceptor is very similar and makes both processes extremely thermodynamically favourable:

$$\text{Glucose} + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta G'_0 = -686 \text{ kcal}$$

$$\text{Glucose} + 4.8NO_3^- + 4.8H^+ \rightarrow 6CO_2 + 2.4N_2 + 8.4H_2O \qquad \Delta G'_0 = -638 \text{ kcal}$$

(Hochachka and Somero, 1984).

Indeed, a study on anoxic maize roots preincubated with nitrate revealed an increased level of nucleoside triphosphates, a lower amount of lactate and a better recovery after anoxia treatment (Fan *et al.*, 1988). Nitrate would accept electrons via mitochondrial respiratory complex 1 (Garcia Novo and Crawford, 1973).

Anaerobically induced proteins

Metabolic changes in response to anoxia would require alterations to the protein expression. One would expect a down regulation of the aerobic metabolism polypeptides and an increased or *de novo* synthesis of enzymes of the anaerobic metabolism. Indeed, with the use of molecular techniques, some dramatic changes in gene expression pattern have been demonstrated. An immediate repression of aerobic protein synthesis during transition to anaerobiosis was observed in maize seedlings roots, as a result of almost complete dissociation of polysomes (Bailey-Serres and Freeling, 1990). This is followed by a transient synthesis of a new set of polypeptides, called transition polypeptides, of molecular weights around 33 kDa (Sachs and Freeling, 1978; Sachs *et al.*, 1980). Then, the synthesis of anaerobically induced proteins (ANPs) commences. ANPs constitute a set of about 20 polypeptides that account for over 70% of the total protein synthesis of the maize roots under anoxia (Sachs *et al.*, 1980). The regulation of the ANPs synthesis appears to occur at both transcription and translation levels. Selective synthesis of ANPs results from induction of the transcription of the respective genes, accumulation of their mRNAs and selective translation of these mRNAs (Sachs *et al.*, 1996).

As it could be expected the majority of anaerobically induced proteins have been identified as enzymes of glycolysis, sugar and sugar-phosphate metabolism, such as sucrose synthase (Springer *et al.*, 1986; Ricard *et al.*, 1991), glucose-6-phosphate isomerase (Kelley and Freeling, 1984a), aldolase (Kelley and Freeling, 1984b), enolase (Bailey Serres *et al.*, 1988) and glyceraldehyde-3-phosphate dehydrogenase (Russell and Sachs, 1989). A second group of ANPs consists of enzymes involved in fermentation, including pyruvate decarboxylase (Laszlo and St Lawrence, 1983; Peschke and Sachs, 1994), alcohol dehydrogenase (Sachs *et al.*, 1980) and lactate dehydrogenase (Good and Paetkau, 1992). The finding of alanine amino transferase among the ANPs in barley roots suggests alanine synthesis as an alternative mechanism of NADH reoxidation (Good and Crosby, 1989). Anaerobically induced superoxide dismutase (Monk *et al.*, 1987) is an example of a protein that will be used upon return to aerobic conditions, when it serves in protection against active oxygen species. In situations where anatomical modifications to provide alternative routes of oxygen supply are a part of anaerobic response, expression of genes involved in such modification would be expected. In maize, anaerobic induction of three genes that seem related to aerenchyma formation have been reported (Sachs *et al.*, 1996).

Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde and functions as a bridge between glycolysis and ethanolic fermentation. In maize, a five to nine fold increase of PDC activity during anoxia was observed, while the transcript level increased by about twenty fold (Kelley, 1989). This increase in activity has been attributed to *de novo* protein synthesis (Laszlo and St Lawrence, 1983). Three

isozymes of PDC and their corresponding genes have been reported in maize. *Pdc1* and *Pdc3* mRNAs significantly increase within 6 h of anaerobiosis, and then remain at very high levels for prolonged period of time, whereas *Pdc2* transcripts do not show much induction in response to oxygen deprivation (Peschke and Sachs, 1994).

Alcohol dehydrogenase (ADH) is probably the most studied anaerobically induced protein in plants. The protein is soluble, found in cytoplasm as either a homo or hetero dimer of randomly associated subunits. For example, in maize two *Adh* genes, *Adh1* and *Adh2*, are present, thus three isozymes, *i.e.*, ADH1-ADH1, ADH2-ADH2 and ADH1-ADH2. The ADH1-ADH1 homodimer is predominant in maize, while the ADH1-ADH2 and ADH2-ADH2 forms are found only in trace amounts (Dennis *et al.*, 1984). In barley, there are three *Adh* genes, giving a total of six ADH isozymes in the cytoplasm of barley cells. The hypoxic and anoxic induction of *Adh* gene is well documented. In the roots of maize, *Adh1* mRNA levels are increased 50-fold by anaerobiosis (Gerlach *et al.*, 1982). Similar results were observed in barley aleurone layers and roots (Hanson and Jacobsen, 1984b), soybean seedlings (Russell *et al.*, 1990), and rice seedlings (Kyozuka *et al.*, 1994). Reflecting the transcriptional and translational induction, ADH activity increases significantly in tissues under limited or no oxygen availability. The increase in *Adh* mRNA levels is detectible shortly after the onset of anaerobiosis, with a maximum level at about 6 hours of anaerobiosis (Gerlach *et al.*, 1982). In barley aleurone layers, ADH activity starts to increase within 6 h of anaerobiosis and reaches its maximum level around 48 h (Hanson *et al.*, 1984b; Taylor *et al.*, 1994), suggesting that alcohol dehydrogenase is a part of both the short and the long term reaction to oxygen deprivation.

Lactate dehydrogenase functions as a tetramer of two randomly associated subunits of 38 and 39 kDa, (Rivoal *et al.*, 1991) encoded by two *Ldh* genes, *Ldh1* and *Ldh2*. Similar to ADH, the levels of lactate dehydrogenase transcripts and activity increase substantially upon oxygen deprivation in barley aleurone tissue (Taylor *et al.*, 1994; Hanson *et al.*, 1984b) and roots (Hoffman *et al.*, 1986), maize roots (Christopher and Good, 1996), and rice seedlings (Rivoal *et al.*, 1991). The half-life of LDH decreases from 240 min in aerobic root extracts to 100 min in anaerobically induced root extracts, suggesting that the increased activity level of LDH is largely attributed to increased level of protein synthesis, which correlates with increased amounts of transcripts (Christopher and Good, 1996). In maize roots, the mRNAs of both *Ldh1* and *Ldh2* reach their peak at approximately 24 h of anaerobiosis, and thereafter, the transcripts decrease as hypoxia continues. LDH activity, however, continues to increase for at least 6 d of anaerobiosis (Christopher and Good, 1996). In barley aleurone layers, LDH activity reaches maximum levels around 48 h and then remains at the same level until 72 h of anaerobiosis (Taylor *et al.*, 1994).

A dramatic induction of haemoglobin transcript, under limited oxygen availability, has been shown in barley aleurone cells (Taylor *et al.*, 1994) and *Arabidopsis thaliana* (Trevaskis *et al.*, 1997). Studies in barley aleurone tissue showed that haemoglobin induction occurs later or at lower oxygen tensions than that of ADH and LDH, suggesting an independent signal transduction (Taylor *et al.*, 1994).

The expression of mitochondrial encoded polypeptides has been found to be affected by anoxia. Mitochondria isolated from aerobic and anoxia-treated rice seedlings

show significant qualitative and quantitative differences in their polypeptides (Couee *et al.*, 1992).

With the characterization of anaerobically induced proteins some questions concerning regulation of their expression arise. Do they all respond to the same signal or are they induced independently? Is the signal transduction uniform for all ANPs ?. Work on promoter sequence analysis, functional analyses of promoters by deletions in transgenic plants and in vivo footprinting were designed to answer some of these questions. Alcohol dehydrogenase genes of maize and *Arabidopsis* served as a model for most of these studies. It has been demonstrated that apart from oxygen deprivation *Adh* is induced by other stresses such as low temperature, heat shock, dehydration and abscisic acid (ABA) (Dolferus *et al.*, 1994a; De Bruxelles *et al.*, 1996). Although both *Adh1* and *Adh2* of maize respond to hypoxia, the signal transduction pathways for them appear to be different since *Adh1* and *Adh2* have different footprints under both aerobic and anaerobic conditions (Paul and Ferl, 1991). Studies on *Adh1* of maize and *Adh* of *Arabidopsis* revealed existence of anaerobic response elements (ARE) in their promoters (Walker *et al.*, 1987; Ferl and Laughner, 1989; Dolferus *et al.*, 1994b). ARE was shown, in *Arabidopsis*, to have a broader function as a general stress response element. Induction by low temperature and dehydration required additional interaction with the G-box motives, that are homologous to the ABA responsive elements (Dolferus *et al.*, 1994b). Thus Adh activation by different environmental stresses requires interaction of different transcription factors and cis-elements within the promoter. *Ldh1* and *Ldh2* may also be differently regulated in maize based on the induction kinetics observed (Christopher and Good,

1996). A gene induction at the transcription level does not always result in higher protein levels. The *Shrunken* (or *sh*) gene, encoding sucrose synthase (SS1), responds to anaerobic stress with a significant increase in mRNA levels (Springer *et al.*, 1986) and no apparent increase in protein (McElfresh and Chourey, 1988), indicating that both transcriptional and translational regulation of ANPs may occur.

Energy metabolism

The complete oxidation of substrate coupled with electron transfer from reduced CoA to oxygen via cytochrome pathway is the most effective process of biological energy production. There seems to be, however, some dispute over the actual amount of ATP produced by this process. The chemiosmoitc theory at best accommodates a value of three ATP formed per electron pair traversing the cytochrome pathway from NADH to O_2, giving a P/O ratio of 3. For electron pairs originating from $FADH_2$ the theoretical P/O ratio is calculated as 2. These ratios are however not found experimentally (Hochachka and Somero, 1984). It has been proposed that, in a living cell the P/O ratio for any given substrate is a measure of not only the cost of ATP synthesis but also the cost of its transport to the cytoplasm against a chemical gradient. Accommodating this fact, theoretical P/O ratios for NADH and $FADH_2$ are calculated as 2 and 1.3 respectively and are close to those measured experimentally (Hochachka and Somero, 1984). The ATP yield of the complete aerobic oxidation of a mole of glucose would then be 25.2 moles ATP, while a calculation with standard P/O ratios gives 38 moles ATP. If palmitate was a substrate, the ATP yields would read 91.8 and 129 per mole.

Certain microorganisms, under limiting oxygen conditions, are able to compensate for the lack of oxygen by utilizing anaerobic respiration with electron acceptors other than oxygen. In energetic terms, the most efficient is nitrate respiration found in denitrifying bacteria. A different set of cytochromes is used for electron transport to nitrate, but the ATP yield per mole of substrate oxidised is similar to that of normal oxidative metabolism (Hochachka and Somero, 1984).

The energetic efficiency of fermentation is, as a general rule, much lower than that of respiration, since only substrate level phosphorylation is available to this type of metabolism. A characteristic of all fermentations is the accumulation of a partially oxidised product. Most commonly utilised, ethanolic or lactic fermentations yield only 2 moles of ATP per mole of glucose fermented. However, some bacteria and lower invertebrates are capable of carrying energetically improved fermentations (Hochachka and Somero, 1984). The most efficient of these is the glucose to succinate pathway producing 4 moles of ATP per mole of glucose and a further oxidation of succinate to propionate giving, in total, 6 ATP per mole of glucose.

Production and utilization of energy are under strict and precise control. Since it was recognized that many enzymes are allosterically affected by either ATP, ADP or AMP and respond to ATP/AMP and ATP/ADP ratios in the cell, (Atkinson and Walton, 1967) proposed the term "energy charge", defined as:

$$\text{Energy charge} = \frac{\text{ATP} + \frac{1}{2}\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

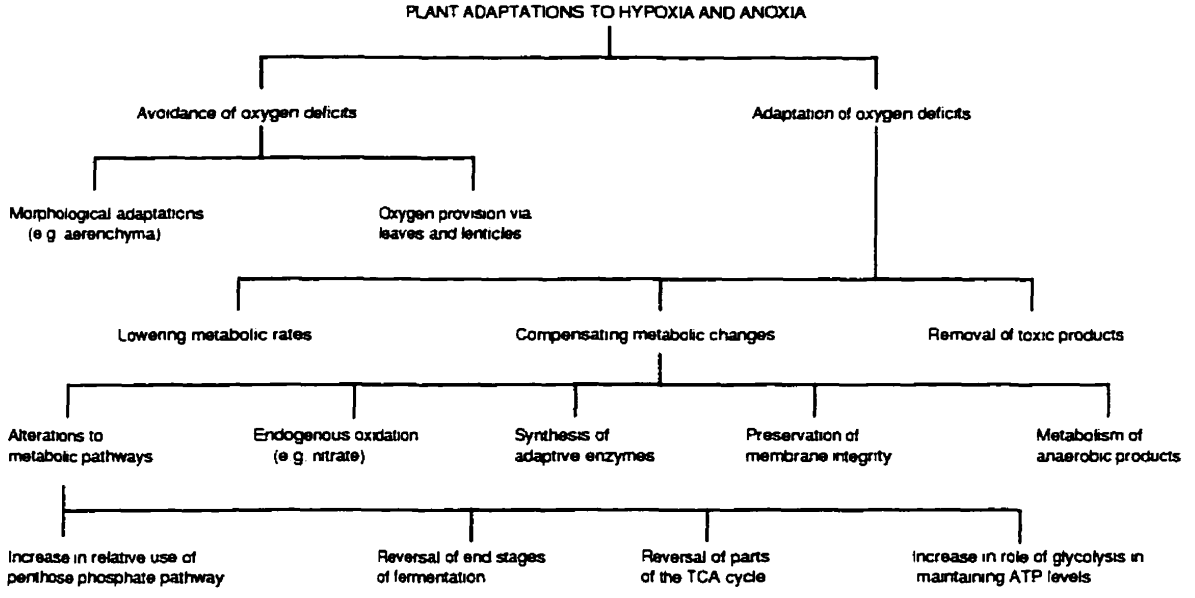
to assess the energy state of adenylate systems. The energy charge would be 1 if all adenylates were ATP and 0 if they were all AMP. With a decreasing energy charge, the

ATP generating enzymes become more active and the activity of ATP utilizing enzymes decreases. Under physiological conditions, the energy charge in plant cells is about 0.8 to 0.9 (Pradet and Rayimond, 1983). Under anoxia or hypoxia, ATP production through oxidative phosphorylation is limited due to insufficient oxygen, and energy charge may drop significantly (Pradet and Rayimond, 1983). Subsequently, the rate of glycolysis increases and fermentative pathways are activated to generate ATP. Similarly, in most fast growing tissues in plants, *e.g.*, root tips, high ATP utilization decreases the ATP/ADP ratio and energy charge (Douce, 1985), which in turn can increase respiration in order to match the energy requirement.

Tolerance of anaerobic stress in plants

The capability of anaerobic synthesis of ATP is the key to the survival of an organism in an oxygen-deprived environment. This in itself, however, may not always be enough for tolerating of long term anaerobiosis. Tolerance of anaerobiosis is usually measured by the length of time an organism or its tissue can be placed under anaerobic conditions and be able to continue growth upon return to air (Crawford and Braendle, 1996).

Figure 3. Plant adaptations to hypoxia and anoxia (from Crawford & Braendle, 1996)



The strategies of tolerance are numerous and may involve several mechanisms (Fig. 3). First, is the avoidance of stress by creating alternative ways of oxygen supply to the affected tissues. Formation of aerenchyma in roots facing oxygen deprivation is a good example of such strategy. It is utilised by various plants ranging from monocotyledonous species such as rice, maize and *Echinochloa* to dicotyledonous plants like waxapple (Lin, 1992). It has been found that the aerenchyma formed within a root indeed improves the oxygen supply to the root tissues (Drew *et al.*, 1985). Although rice, maize and barley can all develop aerenchyma, its capacity for aeration of the root system is ten times greater in rice than barley and four times that found in maize (Perata and Alpi, 1993). Some plant species, like cucumber or *Pisum sativum*, are able to transport oxygen through leaves for root respiration in an O_2-deficient incubation solution (Yoshida and Eguchi, 1994).

Biochemical adaptations are based on alterations to metabolism (discussed earlier), however, some provisions must be made if tolerance of long term anoxia is to be achieved (Hochachka and Somero, 1984). First, the substrate must be available in amounts sufficient to meet the energy needs of tissues under anoxia. In plants, this can be provided if the photosynthetic parts are operating normally. Sucrose synthase plays a special role in plants since it not only breaks down sucrose for glycolysis, but also plays a role in phloem loading and unloading, thus in transport of substrate to the affected tissues (Martin *et al.*, 1993).

Secondly, provisions must be made to store, recycle or minimize productions of noxious fermentative products. There is an ongoing debate on the toxicity of ethanol to plants. Although ethanol toxicity has been shown in a few plant systems (Perata *et al.*, 1986), other experimental data supports the hypothesis that ethanol, in physiological concentrations, does not play a major role in inflicting anoxia-related injuries (Jackson *et al.*, 1982). It has been demonstrated that *Adh1*-null mutants of maize that produce less ethanol than the wild type are more sensitive to anaerobiosis than the wild type plants (Kennedy *et al.*, 1992). In this case, however, the lack of fermentation would negatively affect ATP production. Rice plants depend almost exclusively on ethanolic fermentation for anaerobic ATP synthesis (Cobb and Kennedy, 1987; Fox *et al.*, 1994), while another highly anoxia-tolerant plant, *Echinochloa crus-gali*, uses this pathway to a far lesser extent (Fox *et al.*, 1994). Movement of ethanol into the surrounding medium has been proposed as a mechanism of avoidance of its accumulation in rice (Fox *et al.*, 1994). It has been proposed that cytoplasmic acidosis may be a cause for anoxia intolerance (Roberts *et*

al., 1984). Cytoplasmic acidification may contribute to cell death by reducing glycolytic flux, destroying of intracellular compart mentation and increasing the rate of ATP hydrolysis due to the activation of acid phosphatases (Roberts *et al.*, 1984). Indeed, plant species exhibiting greater anoxia tolerance (such as *Echinochloa sp.* and rice) undergo little or no cytoplasmic acidosis during anoxic treatment (Kennedy *et al.*, 1992). Some animal anaerobes deal with the anoxia induced acidosis by utilizing fermentative pathways that allow higher ATP/H^+ ratios than classic glycolysis, tolerating proton accumulation by recycling it for further metabolism or excretion and by depressing metabolic rates during anoxia (Hochachka and Guppy, 1987).

Thirdly, provision must be made for re-establishing metabolic homeostasis upon return to normal aerobic conditions. Post-anoxic injury, the damage taking place when tissues that have been exposed to anaerobiosis are restored to air, is one problem that significantly affects the survival of plants or plant tissues after the stress. Post-anoxic oxidation of reduced iron triggers the generation of active oxygen species (superoxide *etc.*), followed by H_2O_2 through the action of superoxide dismutase (SOD). H_2O_2 can then react with ethanol that accumulated under anoxia to generate acetaldehyde (Crawford and Braendle, 1996)as shown.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$$

$$O_2^{\cdot-} + O_2 \rightarrow SOD \rightarrow H_2O_2$$

$$H_2O_2 + C_2H_5OH \rightarrow CH_3CHO + 2H_2O$$

Acetaldehyde, however, may be toxic to plants (Perata and Alpi, 1993). H_2O_2 can also be converted to H_2O in a reaction catalysed by ascorbate peroxidase, with ascorbate as a reductant. Anaerobic stress tolerant species have been found to have higher defence capacities by possessing increased levels of SOD and other anti-oxidants such as ascorbic acid, α-tocopherol and glutathione (Crawford and Braendle, 1996). Induction of ascorbate peroxidase and ascorbate regenerating enzymes monodehydroascorbate reductase and dehydroascorbate reductase, was reported in rice seedlings recovering from anoxia (Ushimaru *et al.*, 1997).

The strong hypoxic induction of nonsymbiotic haemoglobin in anaerobiosis sensitive plant species like barley (Taylor *et al.*, 1994) maize (Silva, 1997) and *Arabidopsis* (Trevaskis *et al.*, 1997), represents a possibility of its involvement in hypoxic response.

Hypoxic acclimation

Low oxygen acclimated root tips of maize seedlings show a significant increase in anoxia-tolerance over non-acclimated seedlings (Johnson *et al.*, 1989). Hypoxia acclimated root tips exhibit higher energy charge (Hole *et al.*, 1992) resulting from a sustained glycolytic rate compared to non-acclimated root tips (Xia and Roberts, 1996; Xia and Saglio, 1992). Cytoplasmic acidosis was less pronounced in the acclimated root tips (Xia and Roberts, 1996; Xia and Saglio, 1992). Interestingly, alanine rather than ethanol or lactate was the predominant fermentative end product retained in the cells of acclimated maize root tips (Xia and Roberts, 1994). Similarly, increased survival under

hypoxia caused by ice encasement was shown in plants that were acclimated by exposure to hypoxia (Andrews and Pomeroy, 1983). Correlated with the ice encasement survival were higher levels of adenylates and ATP observed in acclimated plants compared to non-acclimated. This was attributed to the accelerated rates of glycolysis in the acclimated plants (Andrews and Pomeroy, 1989). Hypoxic acclimation, in these experiments, offers a transition time necessary for the induction and synthesis of the complete set of proteins used in the response.

GENE TRANSFER TO PLANT CELLS

Over thirty years have passed since the first attempts to introduce foreign DNA into maize cells (Coe and Sarkar, 1966). Since then the area of plant transformation has seen exciting progress. These first unsuccessful experiments revealed difficulties of plant cell transformation. The cell wall constitutes a barrier to large molecules, such as DNA (Potrykus, 1991). Other limitations are posed by the competence of the plant cell, as only a certain number of cells are capable of accepting and integrating foreign DNA. If transgenic plants are to be regenerated, the cell's ability to regenerate into a plant becomes a factor that may greatly decrease the chances of success. Highly efficient regeneration systems are, therefore, a must for plant transformation. Since the regeneration of transgenic plants was not a purpose of this thesis, this part of the review will briefly discuss only the methods of plant cell transformation.

Over the years, a number of methods have been presented along with claims of successful transformation. The presentation of data is, however, often unclear and may be

misleading. In order to clarify some of these claims, a set of conditions that should be fulfilled to prove integrative transformation was proposed (Potrykus, 1991). These include: a) controls for treatment and analysis; b) a tight correlation between treatment and predicted results; c) a tight correlation between physical (Southern blot) and phenotypic (e.g. enzyme assay) data; d) a complete Southern analysis; e) evidence of absence of contaminating DNA fragments; f) data that allow discrimination between false positives and correct transformants in the evaluation of phenotypic evidence; g) correlation of the physical evidence and phenotypic evidence with transformation to sexual offspring; h) molecular and genetic analysis of offspring populations. Using the above criteria, only five methods have proved successful for transformation of plant cells.

One of the most successful methods of plant cell transformation is *Agrobacterium*-mediated gene transfer. Since it yielded the first transgenic plant cells (Bevan *et al.*, 1983; Herrera Estrella *et al.*, 1983) and transgenic plants (Gasser and Fraley, 1989), this method has been successfully used to transform a variety of dicotyledonous plants. After initial failure of transformation of cereal plants it was concluded that the cereal cell cultures are not amenable to *Agrobacterium*-mediated gene transfer (Potrykus, 1991). Although the full explanation was never given, it was commonly accepted that the lack of wound response in cereals was the reason for the lack of success with *Agrobacterium* in cereals (Potrykus, 1991). This belief was so strong that even the first reports of *Agrobacterium*-mediated transformation of rice (Chan *et al.*, 1993) were welcomed with certain suspicion. Since then the method has been applied successfully in maize (Ishida *et al.*, 1996) and barley (Tingay *et al.*, 1997). This long awaited success was ascribed mainly to the

supervirulent *Agrobacterium* strains and improvements to cell cultures including an active cell division and efficient regeneration systems. The apparent initial failure of *Agrobacterium*-mediated gene transfer to cereals had directed a lot of effort into development of alternative methods based on the direct physical DNA delivery. Four of these methods have brought success. Whereas the *Agrobacterium*-mediated tDNA integration into a host genome is efficient and well understood little is known about processes of the direct-delivery-mediated integration. It is expected, however, that the integration frequencies are lower with the direct delivery methods.

Cell wall free protoplasts are an easy target for transformation. Foreign DNA can be introduced to protoplasts by treatments such as polyethylene glycol (PEG) and electroporation. Such treatments induce a transient porosity in the plasma membrane through which DNA can enter the cell. This method was first used to transform protoplasts of tobacco (Paszkowski *et al.*, 1984), *Triticum monococcum* (Lorz *et al.*, 1985) and maize (Fromm *et al.*, 1986). Although cereal plant regeneration from protoplasts is often problematic, both PEG and electroporation based methods have yielded transgenic plants (Lazzeri *et al.*, 1991; Rhodes *et al.*, 1988).

Electroporation of tissues and walled cells have met with difficulties due to the presence of cell wall that does not respond to the treatment. Mechanical wounding of tissues and enzymatic digestion of immature embryos and cell suspension of maize, resulted in breaking of the cell wall and uncovering plasma membrane, thus enabling electroporation-mediated transformation of tissues (Laursen *et al.*, 1994).

The development of biolistics or particle gun-mediated DNA delivery (Sanford *et*

al., 1987) was met with a great enthusiasm. Accelerated heavy particles (most commonly gold and tungsten 0.4-0.2μm in diameter) coated with DNA can penetrate plant cell walls and carry foreign DNA into the cell's content. The obvious advantages that made this method so popular include its versatility, because it delivers DNA into virtually any type of cell and tissue. The first to be transformed was soybean (McCabe *et al.*, 1988). However, the transformation of maize cell suspension followed by plant regeneration (Fromm *et al.*, 1990) was a breakthrough that led to the extreme popularity of biolistics in cereal transformation.

Silicon carbide fibre-mediated DNA delivery is a fairly inexpensive and simple method of transformation. A tissue sample is vortexed with silicon carbide fibres in a solution of DNA. Silicon carbide fibres are single crystals with an average diameter of 0.6μm and length varying from 10 to 80μm. They exhibit high tensile strength and break through the cell wall, upon collision, during vortexing. Penetration of maize cell walls was elegantly demonstrated by electron microscopy. The DNA adhered to the fibres surface is transported into the cells. DNA delivery with this method was first demonstrated in cell suspension and calli cultures of maize, tobacco and oat (Kaeppler *et al.*, 1990) and *Agrostis alba* (Asano *et al.*, 1991). Stable transformation was documented in cell suspensions of maize and tobacco (Kaeppler *et al.*, 1992) and *Chlamydomonas reinhardtii* (Dunahay, 1993). Transgenic plants of maize were later regenerated from the cell suspension (Frame *et al.*, 1994). The frequency of transformation, as analysed in maize cells, was 1 to 2 orders of magnitude lower than when the biolistics method is used (Kaeppler *et al.*, 1992). Factors that affect efficiency of DNA delivery by silicon carbide

fibres are tissue to fibre ratio, DNA concentration and vortexing speed and time length. Higher efficiency was obtained with suspension cells than with calli simply because of a larger cell surface area that is exposed to the action of fibres in cell suspension. The type of vortexing was found to have an effect as well. Oscillating vortexes were shown to produce higher DNA delivery frequency, probably through increased collision frequency. A drawback of this method, apart from relatively low frequencies of DNA delivery, may be the potential health risks related to the use of fibres. The high length to width ratio of the silicon carbide fibres is similar to that of asbestos fibres, therefore lung damage and carcinogenesis would is a potential hazard.

GENE SILENCING

With the development of transformation systems for various plant types all the excitement and hopes were concentrated around the practical and cognitive value of introducing foreign genes into plants and the over expression of existing genes by the use of constitutive promoter constructs. The ideas of plant genetic engineering became a reality. It was soon discovered that transformation technology may also be used, or inadvertently lead to the down regulation of gene expression or gene silencing. There are two systems of transformation-induced gene silencing, antisense silencing and cosuppression or sense silencing. Although foreign, non-homologous genes can be inactivated in plants (reviewed by Finnegan and McElroy, 1994, and Matzke and Matzke, 1995), antisense RNA silencing and cosuppression are restricted only to homologous endogenous or previously introduced transgenes. Matzke and Matzke (1995) propose

three hypothetical modes of homologous gene silencing based on different nucleic acid interactions in transgenic plants: a) DNA-DNA interaction of inverted repeats leading to *de novo* methylation, b) DNA-RNA interaction where RNA, in high concentration, associates with DNA leading again to *de novo* DNA methylation, c) RNA-RNA interaction where RNA produced from an endogenous gene and homologous transgene hybridize to form double stranded RNA, which is a subject to degradation by RNA-ases. While the last mechanism can explain antisense RNA silencing none of them fully applies to cosuppression.

Antisense RNA

Naturally occurring or artificially introduced antisense RNA, that is RNA molecules that are complementary to the functional cellular RNA (e.g. mRNA), have been implicated in the control of gene expression in both procaryotic and eucaryotic cells. The mechanism of action of the antisense RNA is based on its hybridization to the complementary RNA molecule. The formation of a duplex prevents further interaction with other molecules, thus inhibiting gene function.

The phenomenon of antisense inhibition was first described in bacteria, where naturally produced antisense RNA serves in regulation of plasmid replication (Tomizawa *et al.*, 1981). In bacteria, plasmid replication begins with the RNA replication primer. A short antisense RNA molecule forms a duplex with that primer, thus preventing plasmid replication (Tomizawa *et al.*, 1981). The mechanism of osmoregulation of *E. coli* OmpF protein involves inducible antisense RNA transcribed from OmpC gene (Mizuno *et al.*,

1984). Naturally occurring antisense RNA was reported active in regulation of expression of fibroblast growth factor in oocytes of *Xenopus* (Kimelman and Kirschner, 1989) and myelin basic protein in mice dendrocytes (Fremeau, Jr. and Popko, 1990). In barley, there have been two antisense RNA transcripts identified, which show a strong but imperfect complementarity to α-amylase mRNA (Rogers, 1988), however, their role has not yet been elucidated.

In experimentally created systems, antisense inhibition of gene expression was first shown in transient expression assays when sense and antisense thymidine kinase constructs were co-injected into mouse cells (Izant and Weintraub, 1984). When cells that were previously transformed with a constitutive antisense construct were injected with a sense construct a similar inhibition was observed (Izant and Weintraub, 1984). In plants, transient expression of chloramphenicol acetyl transferase (CAT) constructs was demonstrated when both sense and antisense constructs were introduced into carrot cells. Transient expression of the CAT gene was inhibited up to 95% when both constructs were co-expressed (Ecker and Davies, 1986). A stably transformed system was developed in tobacco (Rothstein *et al.*, 1987). Plants constitutively expressing a bacterial nopaline synthase (NOS) gene were transformed with an antisense construct that contained two thirds of the NOS coding sequence under the control of 35S promoter. A decrease of NOS activity by a factor of 8 to 50 was observed, depending on the developmental stage and growing conditions. Later, in experiments with antisense constructs covering different parts of the NOS gene, it was demonstrated that the degree of NOS inhibition varied greatly depending on the construct used (Sandler *et al.*, 1988). The differences in the level

of inhibition by different constructs were ascribed to the differences in stability of respective RNA transcripts.

The first endogenous gene to be silenced by antisense RNA was chalcone synthase (CHS) in *Petunia hybrida* and *Nicotiana tabacum* (Krol *et al.*, 1988). Plants were transformed with a constitutive construct containing the entire *Petunia* CHS coding sequence in the antisense orientation. Chalcone synthase is an enzyme of the flavonoid biosynthesis pathway and the inhibition or lack of its activity can be easily observed as a discolouration of flower petals. Three different spatial types of discolouration were observed among transformants suggesting that the expression of the antisense construct may depend on the site of integration in the plant's genome. Inhibition of the endogenous tobacco CHS, 80% homologous to the *petunia* gene, with the same construct indicates that full homology is not necessary for the antisense effect (Krol *et al.*, 1988). Similarly, the expression of CHS-A antisense construct inhibits the endogenous CHS-A and related CHS-J in *petunia* (Krol *et al.*, 1990b). Numerous experiments followed these pioneer works, including the most famous inhibition of polygalacturonase in tomato (Smith *et al.*, 1988). Longer lasting tomato fruits, with antisense inhibited polygalacturonase gene, are now commercially available.

Cosuppression

Cosuppression, the phenomenon of coordinate inactivation of a transgene and homologous endogenous gene, or multiple homologous transgenes, in the sense orientation (Krol *et al.*, 1990a) has caught plant scientists by surprise. In the rush for the

introduction of foreign genes into plants, seeking new methods of transformation and trying new genes and species, cosuppression of the genes has been, for many years, unnoticed. The inactivation of a transgene and homologous endogenous gene came unexpectedly as an opposite to anticipated result. In 1990, the first reports of cosuppression (Krol *et al.*, 1990a) (Napoli *et al.*, 1990) came from experiments with pigmentation genes in *petunia*. *Petunia* plants were transformed with dihydroflavonol 4-reductase (DFR) and chalcone synthase (CHS), genes of flavonoid pathway, in order to over express their products. In contrast to expected stronger pigmentation, a discolouration of flowers was observed in 42% of transformants as the result of inactivation of both trans and endogenous DFR and CHS genes. The significance of this phenomenon is recognized by both applied and basic research. For applied science it represents the danger of rejecting transgenes by plants and directs research towards the search for methods of avoidance. For basic research, on the other hand, cosuppression may be a good system for studying gene-gene interactions and has become a field for exploration.

Cosuppression has been, so far, noted only in plants (Finnegan and McElroy, 1994). The question whether this phenomenon can occur in animal cells will have to be answered. The main body of transformation work in plants comes from the *Petunia*, *Nicotiana*, *Arabidopsis* and *Lycopersicon* genera , therefore it is natural that this new phenomenon was first observed in these platns. For the same reason, probably, co-transformation is associated with *Agrobacterium* mediated transformation. Yet, the question remains whether cosuppression is reserved only to certain groups of plants. This

question should soon be answered with the recent, rapid development of successful transformation methodology for cereals.

The phenomenon of cosuppression, has been already noted for a variety of genes (Angenent *et al.*, 1993; Brusslan *et al.*, 1993; Seymour *et al.*, 1993) suggesting that it is a general feature of transgene performance in plants (Blokland *et al.*, 1994). It is puzzling why cosuppression is observed only in limited number of primary transformants (usually 2 to 50%). Similar to the antisense silencing, the level of suppression varies among and within transformants (Napoli *et al.*, 1990; Krol *et al.*, 1990a). In experiments with flavonoid genes (CHS, DFR) variegated pattern of suppression was described. This mosaicism of cosuppression is now thought to be a general feature of the phenomenon(Blokland *et al.*, 1994). The reasons for this variability are not yet understood. Cosuppression is strictly homology dependent. Not always the complete gene is required for elicitation. Truncated and promoterless genes are as active in co-suppressing as the full length ones (Blokland *et al.*, 1994). The cosuppression of polygalacturonase and pectin esterase genes in tomato was even achieved when their homologous genes were introduced in a single chimeric construct (Seymour *et al.*, 1993).

The molecular mechanism of cosuppression has not yet been fully understood. To date, both transcriptional (Brusslan *et al.*, 1993), and postranscriptional (Blokland *et al.*, 1994) inactivation were found. Cosuppression is not a uniform phenomenon. There is a substantial variability among reported cases, therefore there is no single mechanism able to clarify molecular events leading to gene silencing . The common feature of all reports is the involvement of homologous DNA sequences and all hypotheses must include their

interaction either via DNA-DNA mode or through interaction between genes and their products. So far the best documented, case is silencing of flavonoid genes in *Petunia* (Blokland *et al.*, 1994), and the hypothesis that cosuppression results from increased RNA turnover, seems to explain more than one example (Carvalho *et al.*, 1992). It may be questioned, though, by the fact that promoterless gene can also elicit cosuppression.

Inactivation by cosuppression may be a problem in cases where over expression of an endogenous gene is required. This problem can be avoided by selection of transformants tolerating extra copies of homologous genes. As it was shown by many studies (reviewed by (Finnegan and McElroy, 1994; Matzke and Matzke, 1995) cosuppression concerns only a percentage of transformants. A substantial number of transformants, in each report, can tolerate elevated expression of introduced transgenes.

III. MATERIALS AND METHODS

MAIZE CELL CULTURES

Maize (var. Black Mexican Sweet) (BMS) cell suspension was produced from callus provided by Plant Biotechnology Institute, Saskatoon. Calli were broken down to small aggregates with a glass rod and suspended in MS (Murashige and Skoog, 1962) (Appendix I) medium supplemented with thiamine 0.5 mg/litre, L-Asparagine 150 mg/litre, 2,4-D 2 mg/litre and sucrose 20 g/litre. Medium was prepared as 10 times concentrate and stored frozen for up to 60 days. The cell suspension cultures were carried in 250 ml flasks containing 50 ml of medium, on a gyratory shaker at 150 rpm, at the constant temperature of 25°C. The cells were subcultured every seven days by transferring 2 ml (sedimented cell volume) of cells with 5 ml of conditioned medium to flasks containing 45 ml of freshly prepared medium. All media and glassware were sterilized by autoclaving and all handling procedures performed under sterile conditions.
Culture growth was measured by sedimentation in 25 ml graduated pipettes.
Cell samples were collected by filtration. Cell samples used for adenylate measurements or metabolite fractionation were frozen in liquid nitrogen immediately after collection.

HYPOXIC STRESS TREATMENT

Hypoxic stress was applied by replacing air in the culture flasks with nitrogen and closing these flasks with rubber stoppers, otherwise the culture flasks were closed with caps allowing for free exchange of air. The stressed cultures were kept on the gyratory shaker, under the same conditions as the normally grown maize cells.

oxygen dissolved in medium. Silicone stoppers are known to leak oxygen, but the absolute anoxia was not intention of this treatment.

GENETIC TRANSFORMATION OF MAIZE CELLS

Construction of transformation vectors

*Sal*I/*Not*I digested and end-filled barley haemoglobin cDNA was cloned into *Bam*HI digested and end filled pAHC17 plasmid (Christensen and Quail, 1996) in the sense antisense orientation to generate pAS1 (sense) and pAS2 (antisense) plasmids. A 1.35 kb *Eco*RI fragment of pDB1 plasmid, containing 35S promoter-bar gene-35S terminator cassette was end filled and ligated with *HindIII* synthetic linker (5'CAAGCTTG3') before insertion into *HindIII* digested pAS1 and pAS2.

All fragments were recovered from 1% agarose gels either by electroelution followed with double phenol/chloroform extraction, or with the use of Gene-clean kit (Life technologies) used as instructed by manufacturer. All cloning procedures were carried out as described by (Sambrook *et al.*, 1989). All plasmids DNA were produced in *E. coli* DH5α cells (Life technologies). Competent cells were transformed as instructed by manufacturer (38 °C heat shock) and the transformed colonies selected on LB (Luria Bretani) medium supplemented with proper antibiotics.

Plasmid DNA

The pDB1 plasmid DNA (Becker *et al.*, 1994) was provided by prof Horst Lörz, University of Hamburg. The pAS1 and pAS2 vectors were constructed (see results). All vectors were prepared from *E. coli* cultures by lysis by alkali and purified by equilibrium

centrifugation on CsCl-Ethidium bromide gradient(Sambrook *et al.*, 1989).

DNA delivery

DNA delivery was conducted essentially as described by Kaeppler *et al.*, (1990). Silicon carbide fibres (Silar SC-9, ARCO Metals, average diameter 0.6 μm and length 10-80 μm) were handled with care to avoid inhalation. Dry fibres were handled only in a vented flow hood, and when possible, fibres were used in liquid suspension. Fifty mg of dry fibres were autoclaved in a 1.5 microcentrifuge tube with a punctured lid, and wrapped in aluminium foil. Then sterile water was added to make up 5% w/v suspension. The tubes were vortexed to suspend fibres. Fibre suspensions were pipetted with 200 μl tips that were cut to create larger openings. Immediately before use, the tube was vortexed again as the fibres settle quickly.

An aliquot of plasmid DNA was combined with an aliquot of 5% suspension of silicon carbide fibres in a 1.5 ml microcentrifuge tube. The suspension was briefly vortexed. Maize suspension cells were harvested by vacuum filtration, rinsed with fresh medium, and approximately 300 μl of packed volume of cells was added to each tube containing plasmid DNA and fibre suspension. Fresh culture medium (200 μl) was added to the mixture. The tubes were vortexed in inverted position for 10 sec then returned upright and vortexed at top speed with a Fisher Genie II mixer. All procedures were performed aseptically.

Experimental controls were replicated samples consisting of : a) cells + medium, b) cells + medium + DNA, c) cells + medium + silicon carbide fibres.

Selection of stable transformants

Samples of maize cells treated with plasmid DNA and silicon carbide fibres, and control samples were transferred into 60 x 20 mm disposable Perti plates, and 3 ml of medium was added tc each plate. Two days later cells from a single treatment were divided into three equal portions, and each portion was evenly distributed over a filter support paper (7 cm in diameter, Whatman 1) overlying 50 ml of selection medium in a 100 x 25 mm disposable Petri plates. Medium was prepared without L-Asparagine and contained the herbicide BASTA (Hoehst) in concentration of 5 mg/litre of active ingredient, phosphinothricin. Selection medium was solidified with 0.2% Phytagel (Sigma). Support filter papers with cells were transferred to the fresh selection media every 14 days. Putatively transformed cell colonies, growing in the presence of phosphinothricin were observed after 5-6 weeks of selection. When the resistant colonies had grown to about 0.5 cm in diameter they were placed directly on the selection medium and subcultured thereafter onto fresh selection medium. Larger colonies (about 2-3 cm in diameter) were divided in half and the cells used for DNA analysis (Southern, PCR).

Histochemical GUS expression assay

Following vortexing, the contents of each tube were transferred to 60 x 20 mm disposable Petri plates 1.5 ml of fresh medium was added and each plate was sealed with Parafilm. Plates were shaken on a gyratory shaker at 50 rpm at 25°C for 48 hours. After the incubation period histochemical assays of each sample were performed by adding 1.5 ml of histochemical β-Glucuronidase assay solution containing 1 mg/ml of X-gluc in 50

mM $NaPO_4$, pH 7.0 (Jefferson, 1987). X-gluc was first dissolved in dimethyl formamide (1mg/10 μl).The plates were incubated for 48 hours at 25°C. Transient expression events (blue cells or blue cell aggregates) were identified visually under a binocular microscope and counted.

PCR REACTIONS

All PCR amplifications were performed in a 50 μl reaction volume. Reaction mixtures contained reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each dNTP, 1 μM of each primer, template DNA (5 ng plasmid or 1 μg genomic DNA) and 1-2 U of Taq DNA polymerase.

Typically, DNA was amplified in 25-30 cycles of denaturation, annealing and polymerization under the following conditions: denature at 94°C for 1 min (first cycle only), denature at 92°C for 30 sec, anneal at 60°C for 30 sec, extend at 75°C for 1.5 min. Primers used:

a) HBF -haemoglobin forward primer 5'GCCCACTTACGAGAACCAAA3', homologous to the 5' end of barley Hb cDNA.

b) HBR-haemoglobin reverse primer 5'ACGGGTGTTCATTTATACAGGC3', identical to the 3' end of barley Hb cDNA.

c) UBIF-maize ubiquitin (Ubi1) promoter forward primer 5'GATGGCATATGCAGCAGCTA3', homologous to the 3' end of the promoter.

SOUTHERN BLOTTING

Genomic DNA isolation

Genomic DNA was prepared from maize cells essentially as described by Murray and Thompson (1980). Maize cells were frozen in liquid nitrogen and stored in a deep freezer at -70°C. Frozen cells were ground to a fine powder with a mortar and pestle containing liquid nitrogen. For every 5 g weight of fresh material, 15 ml of 60°C pre-warmed cetyltrimethylammonium bromide (CTAB) buffer (see Appendix II) was added and incubated in a 50 ml Corning tube in 60°C water bath for 1 h. The extraction mixtures were gently mixed by inverting the tube every 15 min. An equal volume of chloroform (24 chloroform : 1 isoamyl alcohol, v/v) was added, gently mixed, and centrifuged at room temperature in a SS-34 rotor at 700 rpm for 10 minutes. Aqueous phase was recovered and chloroform extraction repeated once more. A 0.4 volume of 5 M ammonium acetate was added to the aqueous phase followed by 2 volumes of isopropanol. The samples were left for 15 min at room temperature, and the precipitating DNA was recovered by centrifugation. Proteins are not precipitated by isopropanol in the presence of 2 M ammonium acetate. The pellet was washed (2-3 times) with 70% ethanol and resuspended in TE buffer (see Appendix II). RNA was by treatment with RNA-ase. Five hundred micrograms of RNA-ase A (previously boiled for 10 min to eliminate any trace of DNA-ase) was added, prior to 60 min incubation at 37°C. Protein was removed by repeated phenol-chloroform extractions (Sambrook *et al.*, 1989). Equal volume of phenol (Tris-HCl saturated, pH 7.5) was added, the solution was vortexed and centrifuged at 4000 rpm (SS-34 rotor) for 5 min. The aqueous phase was extracted once with phenol:chloroform

(1:1) and once more with chloroform (24 chloroform : 1 isoamyl alcohol, v/v) and centrifuged at 4000 rpm (SS-34 rotor) for 5 minutes. DNA was precipitated again with ammonium acetate and isopropanol. DNA pellet was taken up in a small amount of TE buffer (see Appendix II).

Probe preparation

Barley haemoglobin cDNA (isolated from vector) was labeled with digoxigenin-11-dUTP (DIG) in a PCR reaction with the use of the DIG DNA labeling kit (Boehringer Mannheim). Primers HBF and HBR were used in amplifications. Reactions were carried out according to manufacturers instructions using 1:4 DIG-dUTP:dTTP ratio.

DNA hybridization

Ten micrograms of DNA were digested overnight with 2 µl of desired restriction endonuclease (*SalI*) and proper amount of buffer (One-Phor-All, Pharmacia) in 30 µl reactions. Electrophoresis was carried out in a 1% (w/v) agarose gel with TAE buffer (see Appendix II). The DNA was denatured by soaking gels in 0.2 M HCl followed by incubation in the denaturing solution (1.5 M NaCl, 0.5 M NaOH). Neutralization was obtained by incubation in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.0). The gels were capillary blotted onto nylon membranes, Hybond-N^+ (Amersham), with 20 x SSC for 12 h at room temperature.

The membranes were prehybridized in the hybridization buffer (6 x SSC, 5 x Denhardt's solution, 1% SDS, w/v, and 100 µg/ml salmon sperm DNA) (see Appendix II)

for SSC and Denhardt's) at 65°C for 2 h. Then, DIG-labelled probe was added to the hybridization solution and incubated at the same temperature for 14 to 16 h. The membranes were then washed at 65°C for 30 min in 2 x SSC plus 0.5% (w/v) SDS, then 20 min in 2 x SSC plus 0.1% (w/v) SDS and 5 to 10 min in 0.2 x SSC plus 0.1% (w/v) SDS at 65°C.

Chemiluminescent detection

Chemiluminescent detection of hybridization signals was carried according to the instructions of Genius ™ kit (Boehringer Mannheim). After hybridization and post-hybridization washes membranes were equilibrated in the wash buffer for 1 min, then blocked in the blocking solution for 60 min. Anti-DIG-alkaline phosphatase was added in concentration of 1:10,000 and incubation was carried for 30 min. The membranes were then washed with the wash buffer and equilibrated in detection buffer. CSPD (used as alkaline phosphatase substrate) was applied to the membrane surface and membranes were wrapped in plastic foil. Before exposure to X-ray film, membranes were incubated at 37°C for 15 min.

NORTHERN BLOTTING

RNA isolation

Total RNA for northern analysis was isolated from approximately 3 g of fresh weight tissue. The tissue was ground with a mortar and pestle containing liquid nitrogen, and incubated at 60°C in 10 ml of preheated extraction buffer (0.2 M Na-acetate. 10 mM

EDTA, 0.2% SDS, 0.1 M β-mercaptoethanol, pH 5.2) and 10 ml water-saturated phenol. The resulting solution was homogenized at speed 5 with a Polytron homogenizer (Brinkmann) for 1 min prior to 15 min centrifugation at 6000 rpm (SS-34 rotor). The aqueous phase was recovered and extracted with one volume of phenol/chloroform (1/1, v/v). The aqueous phase was recovered again, after centrifugation at 6000 rpm, and extracted with one volume of chloroform (chloroform/isoamyl alcohol, 24:1, v/v). An 8 M LiCl stock solution was added to the collected aqueous phase to make a final concentration of 2 M. Precipitation was carried out overnight at 4°C and pellet sedimented by centrifugation at 10,000 rpm (SS-34 rotor) at 4°C for 30 min. The pellet was air dried and taken up in 400 µl of sterilized water. The RNA was further purified by one phenol/chloroform and two to four chloroform (24 chloroform : 1 isoamyl alcohol, v/v) extractions until no precipitates were formed on the interphase. The aqueous phase was collected, and RNA precipitated with 0.3 M sodium acetate (3 M stock was added to make up the final concentration) and 2.5 volumes of 95% ethanol. Precipitation was carried out at -20°C for at least 4 h, and pellet recovered by centrifugation at 13,000 rpm in the micro-centrifuge at 4°C. The resulting pellet was air dried and dissolved in 20 to 40 µl of autoclaved double-distilled water. Total RNA was quantified spectrophotometrically, according to its absorbance at 260 nm.

Probe preparation

Probes (cDNA inserts isolated from the vectors) were random primer labeled with [α-^{32}P] dCTP according to Sambrook *et al.*, (1989). The radio-labeled probes were

purified by ethanol precipitation with carrier DNA (herring sperm). Haemoglobin probe was barley Hb cDNA (Taylor *et al.*, 1994) Ribosomal DNA probe was used to indicate equal loadings.

RNA hybridization

Unless otherwise indicated, 10 µg total RNA were denatured at 65°C for 18 min in a final volume of 20 µl containing 4 µl formaldehyde and 10 µl formamide and then chilled on ice, electrophoresed with RNA running buffer (see Appendix II) in a 1.25% agarose gel containing 2.2 M formaldehyde and a trace of ethidium bromide. The gels were capillary blotted onto nylon Hybond N^+ membranes (Amersham) with 20 x SSC (see Appendix II) or 0.05 M NaOH for 10 - 15 h. Membranes that were blotted with 20 x SSC were air dried for 1 h before UV cross-linking on a transilluminator for 3 min. For membranes blotted with NaOH, no UV cross-linking was needed. Prehybridization and hybridization were carried out for 2 and 14 to 16 h, respectively, at 65°C in hybridization buffer containing 6 x SSC, 5 x Denhardt's solution, 1% (w/v) SDS, and 100 µg/ml salmon sperm DNA. The membranes were washed at 65°C for 30 min in 2 x SSC, 0.5% (w/v) SDS, then 20 min in 2 x SSC, 0.1% (w/v) SDS and 5 to 10 min in 0.2 x SSC, 0.1% (w/v) SDS at 65°C. The membranes were wrapped and placed in a cassette with enhancing screens (DuPont) and exposed to Kodak XAR-5 film at -75°C for an appropriate time

PROTEIN IMMUNOBLOTTING

Barley haemoglobin antibody

Polyclonal antibodies against recombinant barley haemoglobin were raised in rabbits. Affinity purified and titrated as described by Duff et al. (1998) antibodies were used in protein immuno assays.

Crude protein extracts

Maize cells (2 g of fresh weight) were ground in 3 ml of ice-cold protein extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and freshly prepared 1 mM dithiothreitol [DTT] and 1 mM phenylmethylsulfonyl fluoride [PMSF]) with pre-cooled mortars and pestles. The extracts were centrifuged at 10,000 rpm (SS-34 rotor) at 4°C for 20 min. The supernatants were transferred into a 1.5 ml micro centrifuge tube and centrifuged at 13,000 rpm at 4°C for 30 min. The supernatants were collected and the protein was quantified by the Bradford protein assay (Bradford, 1976) with the Bio-Rad protein kit using a γ-globulin standard curve.

SDS-PAGE, blotting and immunodetection

Soluble protein extracts were denatured in loading buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 3% β-mercaptoethanol and a trace of bromophenol blue) and incubated at 100°C for 5 min. Denatured extracts were loaded onto 15% SDS-PAGE gels according to Laemmli (1970). Proteins were either stained (by Comassie-blue) or electroblotted onto nitrocellulose filters according to Towbin *et al.* (1979). Gels were

soaked together with nitrocellulose filters in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) and blotted in a Pharmacia electroblotting apparatus. Nonspecific binding was blocked by incubation of filters in 3% powdered milk in TBS buffer for at least 60 min. The blocking solution was then poured off, antibody added in 2% powdered milk in TBS and incubated for 90 min. After three washes in TTBS buffer, the membranes were incubated with the secondary antibody (anti-rabbit IgG conjugated with alkaline phosphatase, 1:3000) in 2% powdered milk in TBS buffer at room temperature for at least 1 h. Thereafter, the membranes were washed six times in TTBS , then in alkaline phosphatase buffer with nitroblue tetrazolium (NBT) and bromo-4-chloro-3-indolyl phosphate (BCIP) added to final concentrations of 3.3 mg/ml and 1.65 mg/ml, respectively (Sambrook *et al.*, 1989).

QUANTIFICATION OF HAEMOGLOBIN IN PROTEIN EXTRACTS

The concentration of haemoglobin in the total soluble protein extracts was calculated by densitometric comparison of immunoblots (in four repetitions) of known concentrations of recombinant barley haemoglobin using a Sharp Diversity 1, PDI-325-OE scanner. Haemoglobin concentration was expressed as percentage of the total soluble protein.

EXTRACTION AND PURIFICATION OF HAEMOGLOBIN FROM MAIZE CELLS

All procedures were performed at 4°C, and all chromatographic separations used a Pharmacia FPLC system. All buffers were adjusted to pH and degassed at 20°C. Collected fractions were analysed spectrophotometrically using A_{412}.About 120 g of maize cells were collected by filtration and immediately frozen. Cells were ground, to a fine powder, in liquid nitrogen and suspended in 300 ml of extraction buffer (50 mM Tris-HCl, ph 8.0, 100 mM NaCl, 5% w/v sucrose, 1 mM dithioreitol, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml chymostatin). Lysate was clarified by centrifugation at 27,000 g for 10 min. The supernatant fluid was fractionated with polyethylene glycol 8000. The red coloured (in case of HB^+ cells)10-20% polyethylene glycol pellet was redissolved in 150 ml of buffer A (50 mM Tris-HCl, pH 9.0, 1mM dithioreitol, 1mM EDTA) and the clarified solution applied at a rate of 1 ml/min to 75 ml Q-Sepharose column (Pharmacia biotech. Inc.), preequilibrated with buffer A. After thorough washing at 1 ml/min, the protein was eluted at the same flow rate with a 200 ml linear gradient of 0-500 mM NaCl in buffer A. The fractions eluting at around 150-200 mM NaCl, contained the most haemoglobin, and were pooled and diluted 3 fold. Ammonium sulphate was added to the pooled sample to a final concentration of 30% (w/v) and dissolved. The sample was then loaded onto a 35 ml Phenyl-Sepharose column preequlibrated with buffer A containing 30% (w/v) ammonium sulphate. Proteins were eluted with a 100 ml 30-0% ammonium sulphate gradient at a flow of 1 ml/min. Haemoglobin containing fractions were pooled and dialysed in buffer A and applied to a prepacked 1 ml MonoQ column. Proteins were eluted with a 20 ml 0-250 mM NaCl

gradient at a flow rate of 1 ml/min. Haemoglobin rich fractions were pooled and ammonium sulphate was added to a final concentration of 30% (w/v). Sample was then loaded onto a prepacked 1 ml Phenyl-Superose column equilibrated with buffer A containing 30% (w/v) ammonium sulphate. Haemoglobin was eluted with a 20 ml 30-0% ammonium sulphate gradient. The fractions from Phenyl-Superose column were analysed by SDS-PAGE and the most pure fractions were pooled. Buffer was exchanged to 50 mM HEPES, pH 7.8, using a Centricon 10 concentrator. Sample was stored at -70°C until used.

OPTICAL SPECTRA OF HAEMOGLOBIN

Optical spectra were acquired using a Helwett Packard spectrophotometer equipped with a HP data acquisition system. Deoxy-haemoglobin was prepared by adding a large excess of dithionate to a HbO_2 sample.

FLUOROCHROMATIC (FCR) TEST OF CELL VIABILITY

Maize cells were harvested by filtration and suspended in a fresh medium containing 3% sucrose. Cells were then tested for viability by staining with fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970). Briefly, fluorescein diacetate (stock solution of 2 mg/ml dissolved in acetone) was added (in a drop-wise fashion until a faint white precipitate could be seen) to the cell suspensions. After a 10 min incubation cells were evaluated for fluorescence at 520 nm after excitation by 480 nm light using a Zeiss photo microscope III fitted with epifluorescence optics. Since cells often tend to

form large aggregates they were vortexed at top speed in a 30 ml test tube with a Fisher Genie II mixer to break up some cell clusters. Only the small aggregates (where cells could be counted) were scored evaluated.

ANTIMYCIN A INHIBITION OF RESPIRATORY CHAIN

Antimycin A was added as a 27 mM solution in 2-propanol to give a final concentration of 0.2 mM. After the addition of antimycin A the cultures were either put under hypoxic stress or grown under normal air atmosphere.

OXYGEN UPTAKE

Oxygen uptake was measured polarographically with an O_2 electrode (Rank Brothers, Cambridge, UK) equipped with a 2-pen recorder. 5 ml of fully aerated culture medium and 150 mg of maize cells were placed in the electrode cell. The linear part of recorded curve was used in calculations. For the measurements at low O_2 concentration, the medium was first degassed and equilibrated with 2% O_2 / 98% N_2 gas mixture.

CO2 EVOLUTION

Air in culture flasks was replaced with N_2 and flasks were closed with rubber stoppers. 1 ml samples were collected with an air tight needle and analyzed by gas chromatography

ENZYME ACTIVITY ASSAYS: ADH AND LDH

Alcohol dehydrogenase and lactate dehydrogenase enzyme activity assays were performed essentially as described by Hanson *et al* (1984b). One gram of freshly harvested

cells was ground with sand in an ice-cooled mortar and pestle containing 2 ml of 0.15 M Tris-HCl (pH 8.0), 10 mM dithiothreitol (DTT). The extracts were centrifuged at 10,000 rpm (SS-34 rotor) for 20 min at 4°C, and the supernatant was used in spectrophotometric assays for ADH and LDH.

ADH activity was assayed in the ethanol to acetaldehyde direction monitoring NAD reduction (at 340 nm). Reaction mixture (final volume of 3.0 ml) contained 1 ml of 0.5 M Tris-HCl (pH 9.0), 100 μmoles of ethanol, 3 μmoles of NAD and 100 μl of enzyme extract. The assays were carried out at 27°C.

LDH activity was assayed in pyruvate to lactate direction, monitoring NADH oxidation (at 340 nm). ADH activity in the extracts was inhibited with 4-bromopyrazole, that at concentrations of 10 mM completely inhibits ADH activity (Hanson *et al.*, 1984b). The reaction mixture (final volume of 3.0 ml) contained 1 ml of 0.5 M Tris-HCl (pH 8.0), 400 μg of NADH, 30 μmoles of 4-bromopyrazole, 30 μmoles of sodium pyruvate and 400 μl of enzyme extract. Assays were carried out at 27°C.

Only freshly harvested cells were used in enzyme assays. ADH and LDH activities were reported as $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein [U].

ATP, ADP AND AMP QUANTIFICATION

Enzymatic assays for ATP, ADP and AMP content of cells were carried out as described by Lowry and Passonneau (1972).

Cell extracts

Maize cells were collected by vacuum filtration and frozen immediately in liquid

nitrogen. They were stored at -70°C until used. The cells were weighed and ground with three volumes of 3 M $HClO_4$ in a mortar and pestle placed in a methanol/dry ice bath maintained at -10°C. When the cells thawed (2 M $HClO_4$ freezes at about -12°C) 1 ml of 1mM EDTA was added for each 0.3 ml of $HClO_4$, and the samples were maintained on ice for 5-10 min with occasional mixing. Then, the samples were centrifuged at 13,000 rpm in a microcentrifuge for 10 min at 4°C.

Samples were neutralized to pH 7.0 with ice cold 2 M $KHCO_3$ (about 0.35 volume per volume of extract, pH checked for every sample). $KClO_4$ precipitate was removed by centrifugation at 4°C. The neutralized samples were stored at -70°C until used in enzymatic assays.

ADP and AMP

ADP and AMP were assayed in three consecutive reactions:

AMP + ATP → 2 ADP (1) myokinase

ADP + P-pyruvate → ATP + pyruvate (2) pyruvate kinase

Pyruvate + NADH → lactate + NAD (3) lactic dehydrogenase

Reaction times: Lactic dehydrogenase 1 min; Pyruvate kinase 2-4 min; Myokinase 3-6 min. Reaction mixture contained: Imidazole-HCl buffer, pH 7.0 (30 mM imidazole base, 20 mM imidazole-HCl), 2 mM MgCl2, 75 mM KCl, 150 μM NADH, 300 μM P-pyruvate, lactic dehydrogenase 0.4 U/ml, and neutralized cell extract. Oxidation of NADH measured spectrophotometrically by following absorbance at 340 nm. After the readings had stabilized ADP was measured by the decrease in reading after addition of pyruvate kinase (0.3 U/ml), and then, after addition of ATP (100 μM) and myokinase (0.36 U/ml), AMP

was measured by further decrease in absorbance at 340 nm. Two moles of NAD are produced per 1 mole of AMP or 2 moles of ADP.

ATP

ATP was assayed in two reactions:

ATP + glucose → ADP + glucose-6-P	(1) hexokinase
Glucose-6-P + NADP → 6-P-gluconolactone + $NADPH_2$	(2) glucose-6-P dehydrogenase

Reaction time: Glucose-6-P dehydrogenase 5 min; Hexokinase 4-6 min.

Reaction mixture contained: 25 mM Tris-HCl buffer, pH 8.1; 1 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM NADP; 1 mM glucose; hexokinase (0.28 U/ml); glucose-6-P dehydrogenase (0.36 U/ml) and the neutralized cell extract. ATP was quantified by following increase in absorbance at 340 nm.

C^{14} LABELLING OF CELL METABOLITES

Maize suspension cells, after 10 hours of hypoxia treatment, and air treated cells were transferred into fresh media in which sucrose content was decreased to 0.1% w/v and osmotic pressure maintained by the addition of mannitol. Media were previously saturated with nitrogen gas. C^{14} sucrose (40μCi) was added into each flask and treatments were continued for 60 min. All procedures were performed under a flow of nitrogen to avoid oxygen contamination. Cells were harvested by vacuum filtration, washed with water and immediately frozen in liquid nitrogen.

Extraction and fractionation of metabolites

Metabolites were extracted from maize cells and separated as described by Canvin and Beevers (1998). Briefly, 2 g of frozen sample was ground to a fine powder with a mortar and pestle containing liquid nitrogen and then successively extracted for 10 min periods on the steam bath with 20 ml of boiling 80% ethanol, 20 ml of 20% ethanol, 20 ml of water and, finally, 20 ml of 80% ethanol. The extracts were filtered and combined; the insoluble residue was retained by the filter. The combined extracts were placed under vacuum in a water bath using Rotovapor and taken up in 2 ml of water (18.2 MΩ). The water soluble fraction was separated into three fractions through the use of ion exchange resins. These fractions were acidic (mainly organic acids and sugar phosphates), basic, mainly amino acids, and neutral mainly sugars. The aqueous extracts were passed through a Dowex 1-X10 column (formate, generated from the chloride form with 60 ml of 1 M sodium formate and 30 ml of 0.1 N formic acid) connected in series to a Dowex 50-X8 (hydrogen) column and washed with 120 ml of water. Both columns were 7 x 1 cm. The effluent contained the sugar fraction. The columns were, then, separated and the amino acids were eluted from the dowex 50 resin with 80 ml of 1 N NH_4OH and the organic acids were eluted from the Dowex 1 resin with 80 ml of 4 N formic acid. The acidic fraction was further fractionated with reverse phase chromatography, using Aminex HPX-87H HPLC column and Beckman HPLC pump. Refraction index detector was used to monitor the peaks. Samples were separated using 0.0012 M H_2SO_4 as the mobile phase, with the flow rate of 0.5 ml/min. The fractions (0.25 ml) were collected and radioactivity determined with a Beckman LS1701 scintillation counter.

IV. RESULTS

This section will describe results of experiments relevant to the generation of a transgenic maize cell system for haemoglobin studies, and the effects of expression of haemoglobin in these cells. The results will be presented in two parts. The first part, "Transformation and expression of haemoglobin in maize cells" will be described in the sequence of: a) presence and expression of haemoglobin in maize (var. Black Mexican Sweet) suspension cells; b) production of vectors for genetic transformation of plant cells; c) transformation of maize cells and selection of stable transformants; d) purification of haemoglobin from maize cells. The second part, "Altering haemoglobin levels changes energy status of maize cells under hypoxia" will deal with the effects of haemoglobin expression on culture growth, oxygen uptake, energy status and metabolism of maize cells under the conditions of normal and limited oxygen supply.

TRANSFORMATION AND EXPRESSION OF HAEMOGLOBIN IN MAIZE CELLS

A nonsymbiotic haemoglobin gene is hypoxia inducible in maize (BMS) suspension cells

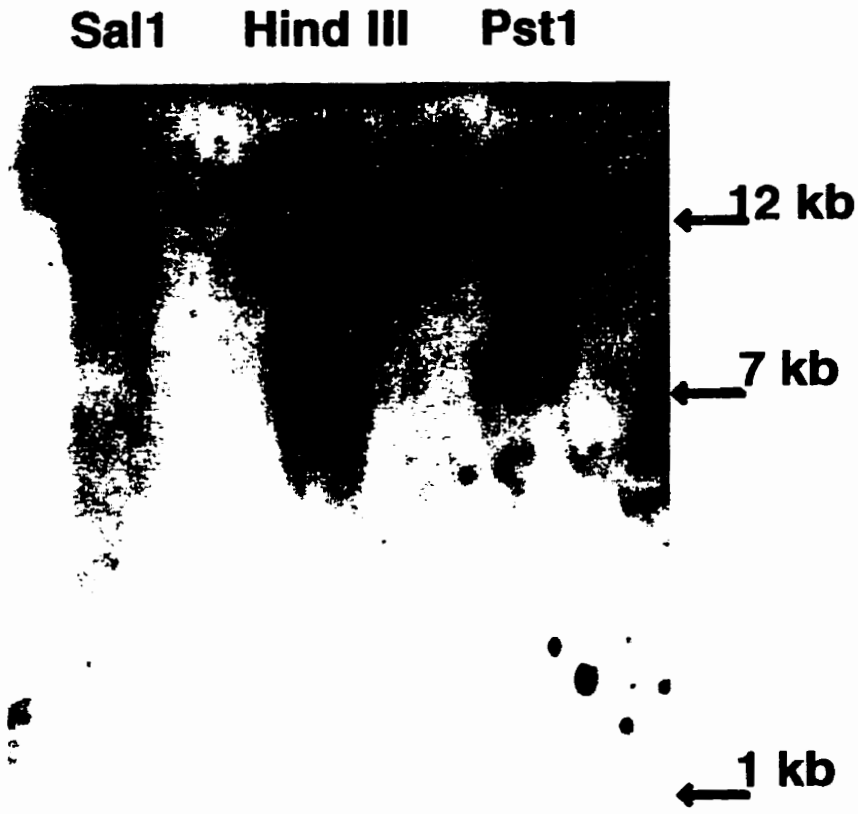
The expression of nonsymbiotic haemoglobins in *Arabidopsis* (Trevaskis *et al.*, 1997), soybean (Andersson *et al.*, 1996), and rice (Arredondo-Peter *et al.*, 1997) has been found to be tissue specific. In the case of barley, maize and *Arabidopsis* hypoxia inducibility of the Hb gene was observed, suggesting that the expression of haemoglobin may be a part of a defence strategy against insufficient oxygen availability.

This thesis seeks a demonstration of the effects of haemoglobin activity in plants. Genetic transformation, with the barley Hb cDNA constructs, has been used to achieve constitutive suppression and over expression of haemoglobin that will, *bona fide*, show the significance of the presence of haemoglobin in a plant cell. Haemoglobin introduced into a cell, through genetic transformation, can function only if it is properly assembled in the cell. If any interaction with other cell compounds is a part of the mechanism of the haemoglobin action, then the introduced protein should be recognized by this compound. Moreover the effectiveness of antisense suppression is strictly homology dependent. For all the above reasons, the plant material used for such experiments should, therefore, possess a functional, hypoxia-inducible haemoglobin gene that shares sufficient sequence homology with the barley Hb to grant cross-hybridization, and be amenable to genetic transformation. The maize (BMS) suspension cultured cells meet all of these conditions.

The presence of a haemoglobin gene in the maize genome has previously been demonstrated by Taylor *et al.* (1994) and its hypoxia inducible expression in maize roots by Silva (1997). Southern blot analysis of the Black Mexican Sweet maize genomic DNA (Fig. 4) shows the presence of a sequence hybridizing to the barley Hb cDNA. The single hybridization band indicates that this sequence exists, most probably, in a single copy.

Figure 4. Southern blot analysis of maize (BMS) genomic DNA probed with barley Hb cDNA.

Maize genomic DNA (30 μg) was digested with *Pst1*, *Hind* III, and *Sal1* endonucleases and probed with barley Hb cDNA. Full length probe DNA was labelled with digoxigenin in a PCR reaction using HBF and HBR primers. Hybridization was at 65°C, 6 x SSC. Final wash was at 65°C, 0.2 x SSC. Hybridization bands were visualized by chemiluminescence, film exposure was 16 h. DNA size markers are indicated on the right. Note that *Pst1*, *Hind* III, and *Sal1* restriction site are not present in the barley Hb cDNA.


Sal1
Hind III
Pst1
12 kb
7 kb
1 kb

To investigate whether haemoglobin can be induced by hypoxia in maize (BMS) suspension cells, the cultures were placed under N_2 atmosphere for various periods of time. Northern blot analysis of the BMS suspension cultured cells (Fig. 5) shows a very low level of expression of haemoglobin message (mRNA) under normal culture conditions and a significant induction in the amount of transcript under the conditions of limiting oxygen. The pattern of induction is similar to that observed in barley aleurone tissue (Taylor *et al.*, 1994; Nie, 1997) and maize roots (Silva, 1997). Induction of Hb transcript is visible within 6 hours of the cell's exposure to hypoxia, reaching maximum at about 12 hours and beginning to decline after about 24 hours of stress.

The induction of haemoglobin under hypoxia was also analysed at the protein level (Fig.6). Antibodies that were raised and titrated against recombinant barley haemoglobin (Duff *et al.*, 1997) were used in protein immunoblot analysis, to investigate whether the same induction can be observed as with the message. The western blot analysis in Fig.6 shows equal levels of haemoglobin polypeptide under normal growth conditions and various lengths of hypoxic exposure. This apparent lack of induction of haemoglobin protein under hypoxia may suggest either a post transcriptional or post translational regulation of Hb expression.

Figure 5. Northern blot analysis of haemoglobin expression in maize (BMS) suspension cells under normal air and hypoxic conditions.

10 μg of total RNA isolated from normally cultured and hypoxia treated BMS cells was probed with barley Hb cDNA. Probe was ^{32}P-labelled by random hexanucleotide priming. The lengths of hypoxia treatment are shown. After autoradiography membrane was stripped and reprobed with ribosomal DNA probe (rDNA) to ensure equal loading. Hybridizations were at 65°C, 6 x SSC. Final wash was at 65°C, 0.1 x SSC. Autoradiography was for 24 hours.

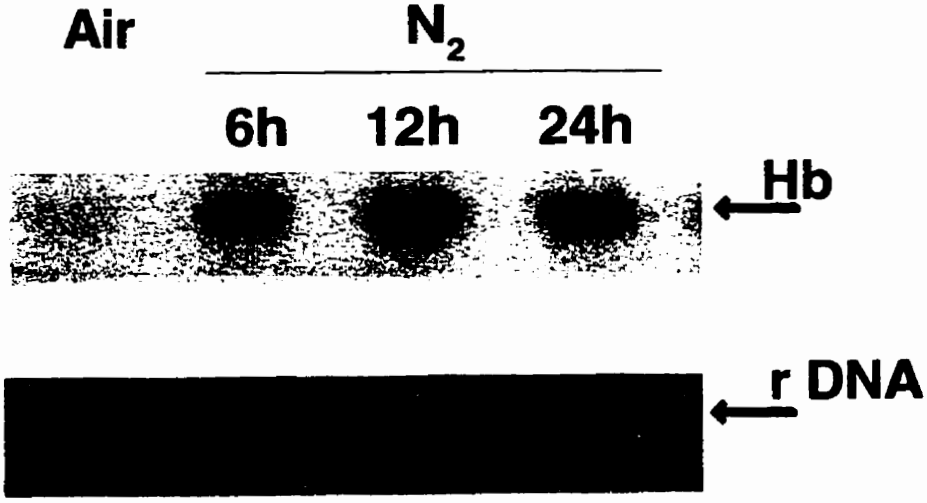
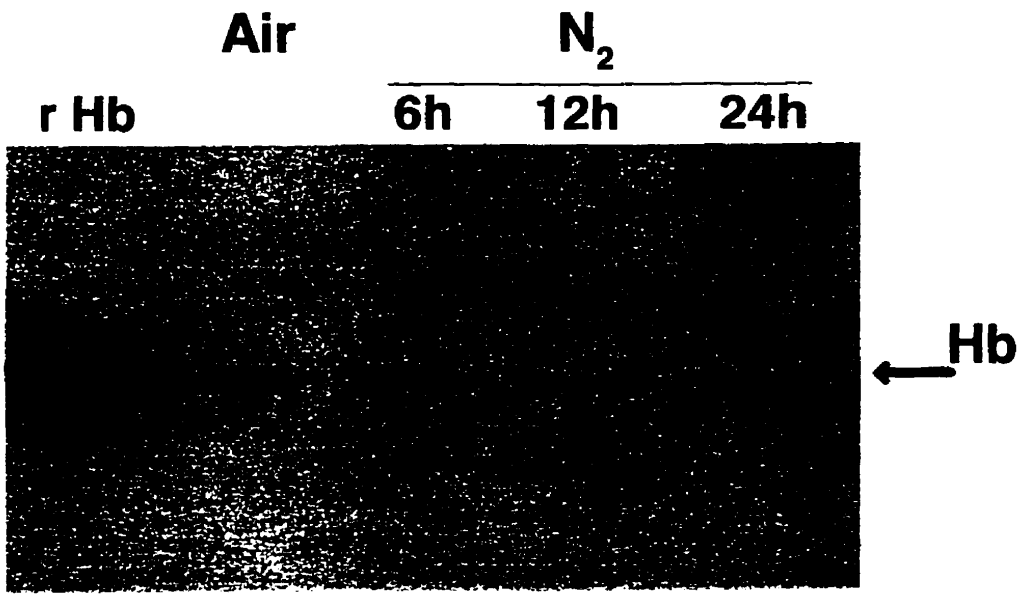

Air
N_2
6h
12h
24h
Hb
r DNA

Figure 6. Protein immunoblot analysis of haemoglobin expression in maize (BMS) suspension cells under normal air and hypoxic conditions.

60 μg of total soluble protein were loaded in each lane. 20 ng of recombinant barley Hb were loaded in the first lane as a size and immunodetection standard.


Air
N2
r Hb
6h
12h
24h
Hb

The observed induction of haemoglobin under low oxygen stress seems to be much higher at the level of mRNA than polypeptide (Fig. 5; 6). Bearing in mind that the antibody used for immunodetection was raised against recombinant barley haemoglobin, this finding may represent a potential problem of lower sensitivity of this antibody against the haemoglobin of maize. The detection of the Hb polypeptide in cells grown under normal air conditions (Fig. 6), together with the barely detectable Hb message, under these conditions, may suggest that the haemoglobin protein turnover is slower than that of its transcript.

These findings, together with reports on transformation of maize cell suspensions (Frame *et al.*, 1994; Gordon Kamm *et al.*, 1990; Kaeppler *et al.*, 1990; Kaeppler *et al.*, 1992) make the BMS maize suspension cell culture a promising material for further research. Moreover, the induction of haemoglobin under oxygen stress in the undifferentiated cells reinforces the hypothesis that the nonsymbiotic haemoglobin may play a fundamental role in the response of plant cells to hypoxia.

Transformation vectors

At the time of these experiments only the direct DNA delivery methods, such as biolistics, electroporation, PEG-induced DNA uptake by protoplasts and silicon carbide fibres-mediated DNA delivery were available for transformation of the cereal plant cells. The plasmid vectors for use with these methods do not require T-DNA flanking sequences. The over expression and antisense silencing of haemoglobin in maize cells demands a strong constitutive promoter for the control of transcription of barley Hb cDNA. A

comparative study of various promoters for transgene expression in monocotyledonous species (Christensen *et al.*, 1992; Taylor *et al.*, 1994; Gallo Meagher and Irvine, 1993) showed that the highest expression level of a marker gene was achieved with maize ubiquitin (Ubi1) promoter (Christensen and Quail, 1996).

In order to produce haemoglobin expression constructs, barley Hb cDNA was placed, in sense and antisense orientation, between the maize ubiquitin (Ubi1) promoter and nopaline synthase (NOS) terminator (Fig. 7). The Ubi1 promoter element contains the Ubi1 regulating sequence together with the first exon and intron of the Ubi1 gene. The first exon of the maize Ubi1 gene does not contain the starting codon (ATG) and constitutes the untranslated mRNA leading sequence. Therefore, the start codon provided by the barley Hb cDNA, in the sense construct (Fig. 7), should be used to initiate translation of the message resulting in the full and unaffected haemoglobin polypeptide sequence.

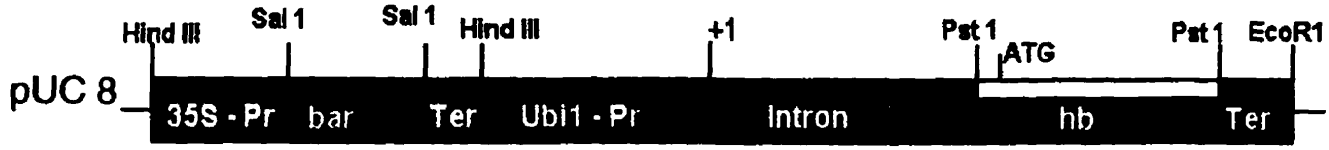
The orientation of barley Hb cDNA in these constructs can be shown by PCR amplifications with UBIF, HBF and HBR primers (primer sequences in materials and methods, and their annealing sites depicted schematically in Appendix III). The HBF is a forward primer identical in sequence to part of the 5' end of the barley Hb cDNA, and the HBR is a reverse primer derived from the 3' end of this cDNA. A PCR reaction with these primers should yield a 870 bp product with both pAS1 and pAS2 used as a template.

Figure 7. Diagrams of transformation vectors.

Barley haemoglobin cDNA was placed in sense (pAS1) and antisense (pAS2) orientation under the control of maize ubiquitin (Ubi1) promoter. The translation start codon in pAS1 is provided by the barley Hb cDNA. The untranslated, first exon of Ubi1 gene is shown in orange. 35S-Pr - CaMV35S promoter; bar - phosphinothricin acetyl transferase gene from *Streptomyces hygroscopicus*; Ter - terminator; Ubi1-Pr - maize ubiquitin 1 promoter; Intron - first intron of maize ubiquitin 1 gene; hb - barley haemoglobin cDNA.

A.

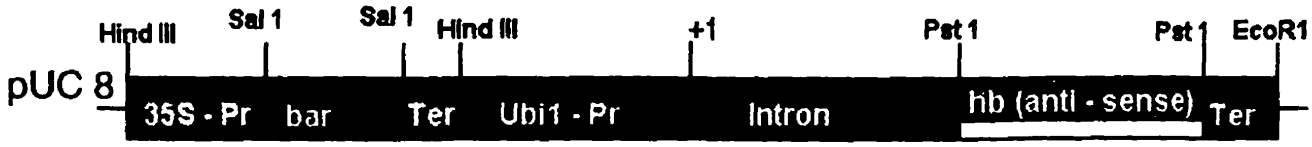
pAS1 (sense)



B.

1Kb

pAS2 (anti - sense)



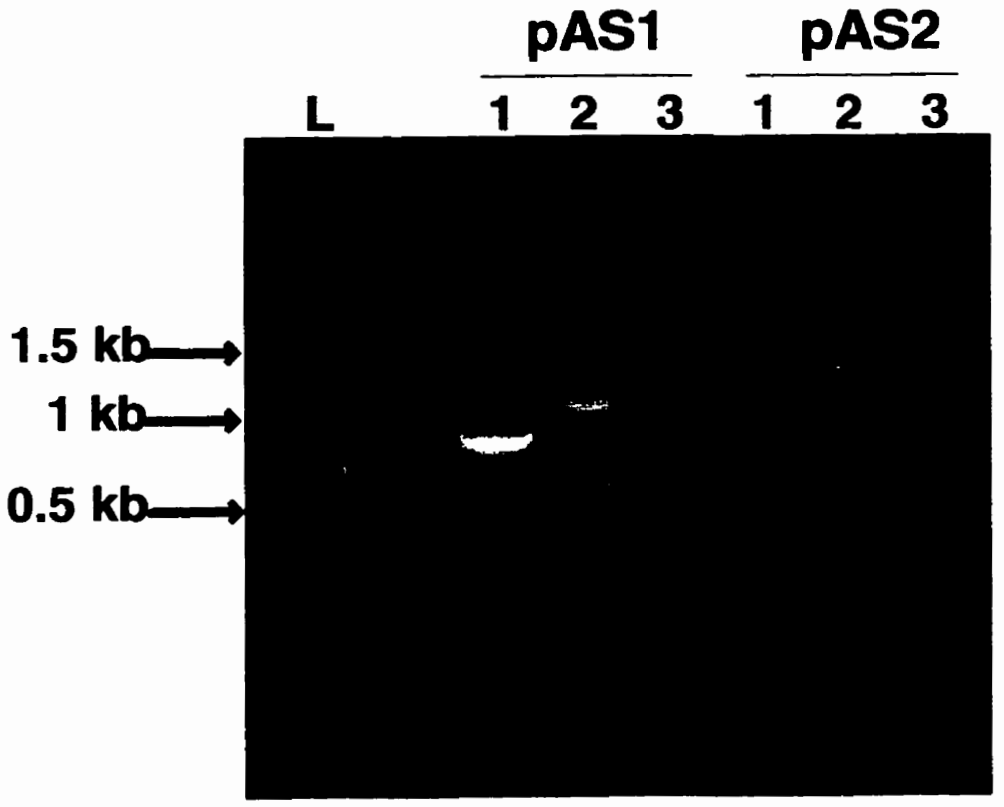
UBF is a forward primer complementary to the 3' end of the first intron of maize Ubi1 gene. A PCR reaction with UBIF and HBR should amplify a 1013 bp product from pAS1 (sense construct) and no amplification product would be expected from pAS2 (antisense construct). Amplification with UBIF and HBF primer pair should yield a 1034 bp product from pAS2 (HBF becomes a reverse primer when Hb cDNA is in the antisense orientation), and no product should result from pAS1 template. Figure 8 shows correct PCR amplification products, confirming the sense orientation of Hb cDNA in pAS1 and antisense in pAS2.

To facilitate selection of transformed cells the *bar* gene, under the control of the cauliflower mosaic virus CaMV 35-S promoter, was cloned head to head with haemoglobin expression constructs in both pAS1 and pAS2 plasmid vectors (Fig. 7). The *bar* gene codes for bacterial (*Streptomyces hygroscopicus*) phosphinothricin acetyl transferase (PAT), whose product deactivates phosphinothricin, an inhibitor of plant glutamine synthase (GS), and therefore conferres resistance to the phosphinothricin or glufosinate ammonium-based herbicides (Eckes *et al.*, 1987).

The pAS1 and pAS2 plasmids can be used to transform a variety of plant species with the use of any direct DNA delivery methods including biolistics, polyethylene glycol, electroporation and silicon carbide fibres. The constitutive activity of maize ubiquitin (Ubi1) promoter has been shown to be 3 to 50 fold greater than that of commonly used CaMV35-S (Christensen and Quail, 1996; Taylor *et al.*, 1993).

Figure 8. PCR determination of the orientation of the barley Hb cDNA in transformation vectors.

DNA of pAS1 and pAS2 plasmid vectors was used as a template for PCR reactions with three sets of primers: 1) HBF/HBR; 2) UBI1/HBR; 3) UBI1/HBF. Lack of a PCR product is expected when both primers anneal to the same strand of template DNA. Expected product lengths for sense orientation are: 870 bp - HBF/HBR; 1013 bp- UBI1/HBR, and for the anti sense orientation: 870 bp - HBF/HBR; 1034 bp - UBI1/HBR.


pAS1
pAS2
L
1
2
3
1
2
3
1.5 kb
1 kb
0.5 kb

Silicon carbide fibres-mediated DNA delivery was selected as a rather inexpensive transformation method. This method was shown effective in transformation of maize suspension cultures (Kaeppler *et al.*, 1992; Frame *et al.*, 1994).

Determination of the DNA delivery parameters

Since the efficiency of DNA delivery and stable transformation varies significantly between the published reports on silicon carbide fibres-mediated plant cell transformation (Kaeppler *et al.*, 1990; Kaeppler *et al.*, 1992; Frame *et al.*, 1994) experiments were conducted to determine the parameters of the most efficient DNA delivery process. The efficiency of DNA delivery with the silicon carbide fibres method depends on the ratio of cells to fibres, length of vortexing time and availability of the vector DNA. To some extent, the effect of increased fibre concentration can be achieved by increasing the vortexing time. Therefore, in this study, the fibre concentration remained constant, and only the effect of vortexing time and DNA concentration on the efficiency of transformation was tested.

To determine the most efficient parameters of DNA delivery with silicon carbide fibres a transient expression assay was applied. The pDB1 plasmid vector (Becker *et al.*, 1994) (depicted schematically in Appendix IV), harboring UidA gene (GUS) under the control of rice actin Act1 promoter, was used for easy detection of transformed cells. Histochemical detection of GUS activity, 72 h after treatment with silicon carbide fibres and plasmid DNA, is shown in Fig. 9. A cluster of blue-stained cells, or a single blue cell,

were counted as one GUS expression unit. The effect of vortexing time of maize (BMS) cells with silicon carbide fibres and DNA on efficiency of the DNA delivery and cell survival of the treatment is shown in Fig. 10. The experiment was performed using 25 μg DNA per treatment as proposed by Kaeppler et al. (1992). The number of GUS expression units increased steeply up to about 85 per treatment by 120 s and continued to increase at a slower pace up to the maximum time tested (240 s) (Fig. 10). Treatment with silicon carbide fibres has been shown to physically damage the cells and affect their viability (Kaeppler *et al.*, 1990; Frame *et al.*, 1994). The cell survival of the treatment decreased continuously, reaching 64% of viable cells at 240 s. These results, presented in Fig. 10, indicate that the 120 s treatment is the most effective in DNA delivery to maize cells, producing about 85 GUS expression units per treatment of 300 μl of cells with 25 μg of DNA and 40 μl of 5% suspension of silicon carbide fibres.

Figure 9. Histochemical detection of GUS activity in maize cells.

The pDB1 plasmid vector harbouring the UidA gene under the control of the rice actin (Act1) promoter (Becker *et al.*, 1994) (Appendix IV) was used in determination of the DNA delivery parameters with silicon carbide fibres. Histochemical staining was performed 72 hours after the treatment. Blue cell colony, as shown, was counted as 1 GUS expression unit.

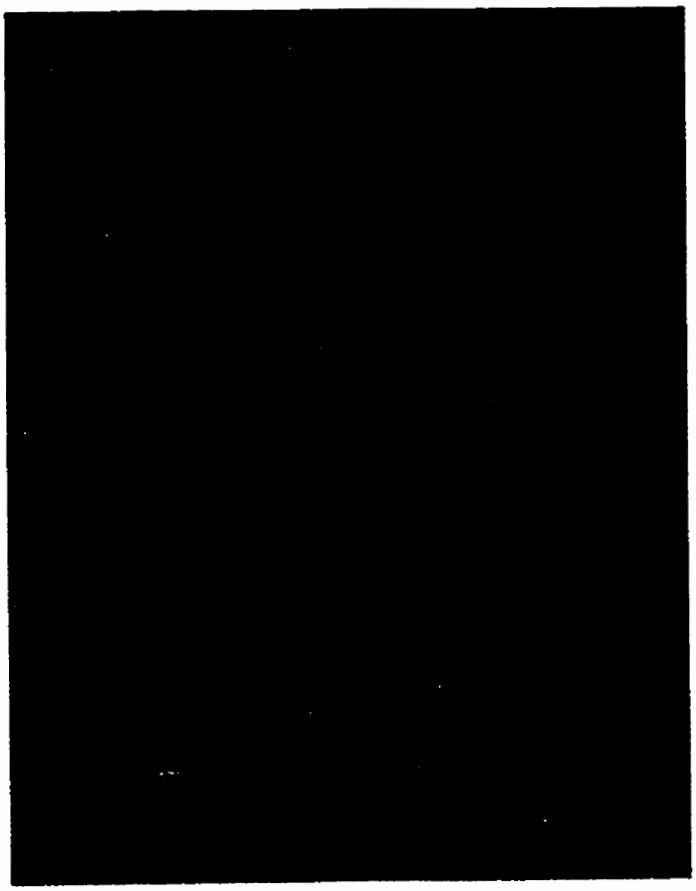
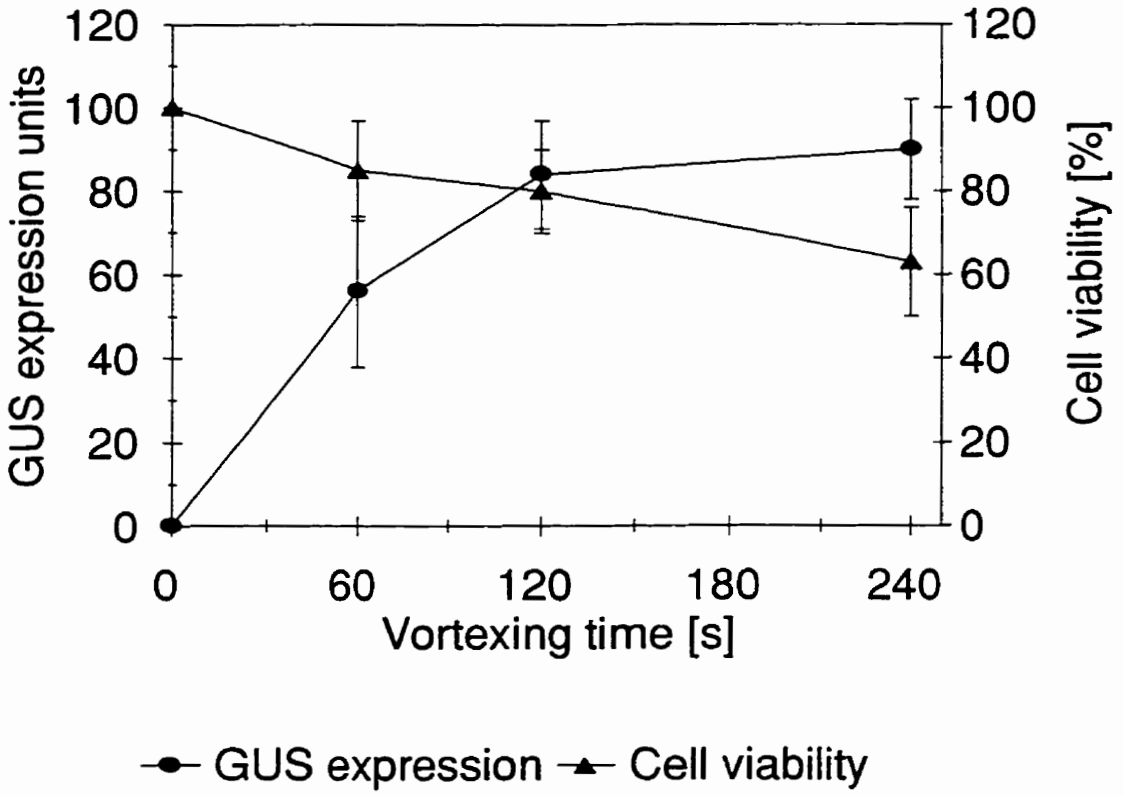


Figure 10. Effects of the length of silicon carbide fibres treatment on cell viability and transient GUS expression.

Treatments were performed with 25 μg of plasmid (pDB1) DNA.

GUS expression was assayed by histochemical staining and cell viability by FCR (fluorescein diacetate) test, 72 hours after treatments. Line bars indicate standard error of three repeats.


120
100
80
60
40
20
0
GUS expression units
120
100
80
60
40
20
0
Cell viability [%]
0
60
120
180
240
Vortexing time [s]
GUS expression
Cell viability

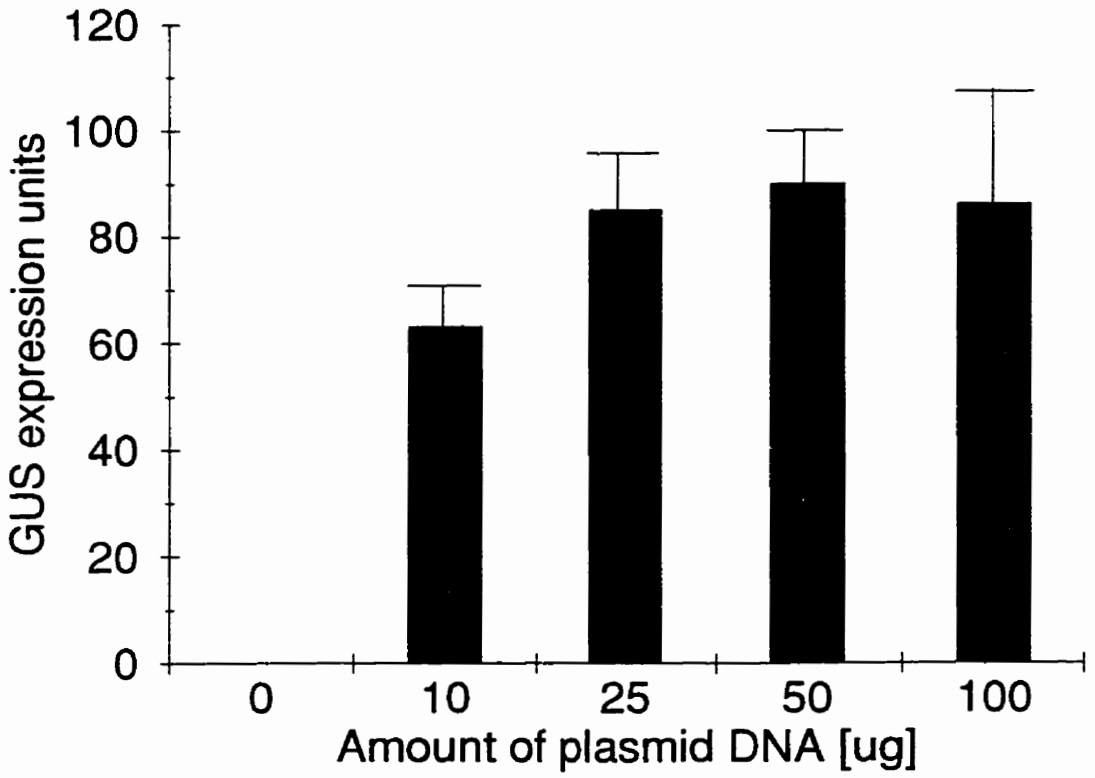
To determine the most effective vector DNA concentration for the silicon carbide fibres-mediated transformation of maize BMS cells, transient GUS expression assays were conducted after the treatments with various amounts of plasmid DNA, ranging from 0 to 100 μg (Fig. 11). The GUS activity was not detected in the cells treated without the vector DNA (Fig.11). With 10 μg of DNA added to the treatment tube, about 60 GUS expression units were counted in the transient expression assay. Increasing the DNA amount to 25 μg produced over 80 GUS expression units per treatment, and further increase did not significantly affect the efficiency of DNA delivery (Fig. 11).

Transformation of maize (BMS) cells with sense and antisense haemoglobin constructs

The pAS1 and pAS2 plasmid constructs were used to transform maize (BMS) suspension cells. The time of vortexing of cells with silicon carbide fibres in DNA solution and the DNA concentration were set at 120 s and 25 μl respectively, according to the results of transient expression assay (Figs. 10 and 11). Apart from these modifications DNA delivery was performed as described by Kaeppler *et al.*, (1990). Twenty independent treatments were performed for each plasmid vector.

Figure 11. Effect of the amount of plasmid DNA on silicon carbide fibres-mediated DNA delivery.

300 μl of maize BMS cells were treated in 1.5 ml Eppendorf tubes with 40 μl of 5% silicon carbide fibres suspension (as described by Kaeppler *et al.*, 1994)and various amounts of plasmid DNA. Transient GUS expression was assayed histochemically 72 hours after treatments. Line bars indicate standard error of three repeats.


120
100
80
60
40
20
0
GUS expression units
0
10
25
50
100
Amount of plasmid DNA [ug]

Selection of stable transformants

Selection of cells treated with pAS1 and pAS2, on media containing 5 mg/l of glufosinate ammonium, resulted in 51 and 63 herbicide resistant colonies respectively, giving a mean of 2.85 resistant colonies per treatment (Table 2). The first resistant colonies were visible after 6 to 7 weeks and the entire selection process continued over 11 weeks after transformation. An example of a glufosinate ammonium resistant maize (BMS) cell colony and dead, susceptible cells is shown in Figure 12.

To determine whether the selected cell colonies were truly transformed with the haemoglobin constructs Southern blot analysis of their genomic DNA with barley Hb cDNA probe was performed (Fig. 13). Genomic DNA, isolated from the selected cells, was digested with Pst1 restriction endonuclease to liberate the 924 bp Hb cDNA fragment (see Fig. 7). The analysis of undigested DNA was not performed for two reasons. First, the genome of the BMS maize cells already contains a haemoglobin gene that hybridizes to the barley Hb cDNA probe (Fig. 4), and therefore all cell colonies would be expected to test positively. Second, the amount of genomic DNA isolated from the small cell colonies would not support two Southern blot experiments. The Southern blot analysis of genomic DNA isolated from the wild type BMS cells, and digested with the Pst1 endonuclease shows a Hb hybridization band at about 10 kb, which can be easily distinguished from the 924 bp band of the transgene Hb.

Table 2. ***Selection of glufosinate ammonium resistant maize cell colonies***

Vector	Number of treatments	Number of glufosinate ammonium resistant colonies	Number of stable transformants
pAS1	20	51	24
pAS2	20	63	38
total	40	114	62

Figure 12. Selection of stable transformants on glufosinate ammonium.

Three days after silicon carbide fibres / DNA treatments, maize cells were plated on Phytagel solidified media, lacking asparagine and containing 5mg/l of glufosinate ammonium (active ingredient of herbicide "Basta", Hoehst). Resistant cell colonies were visible after 6 weeks. Photograph was taken 11 weeks after treatment. Growing, glufosinate ammonium-resistant callus is shown on the background of dead cells.

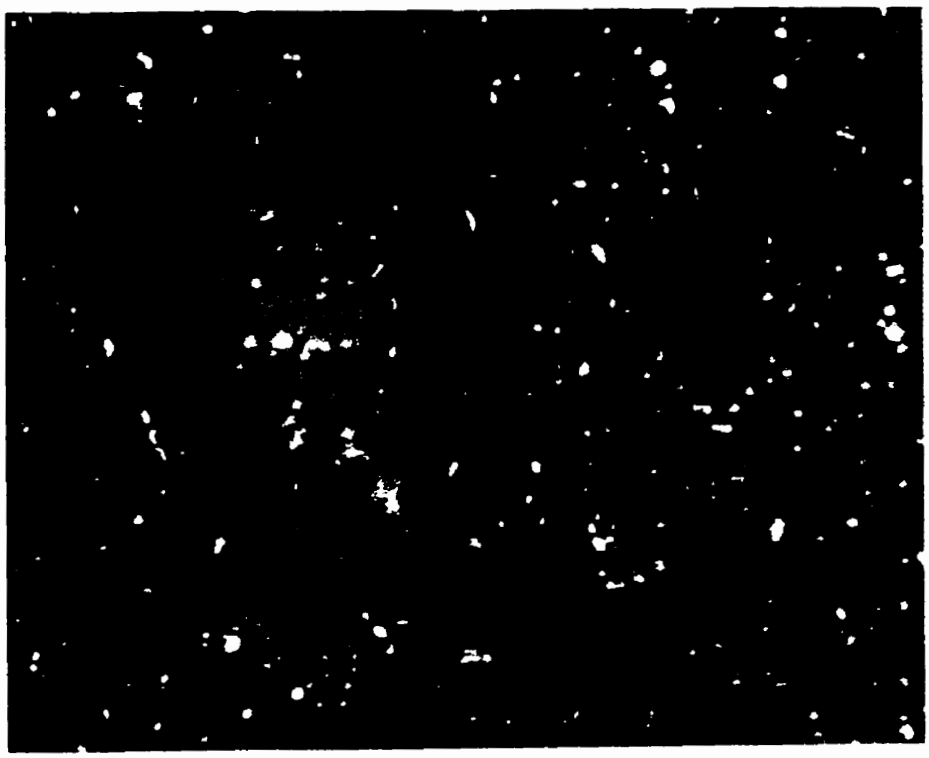
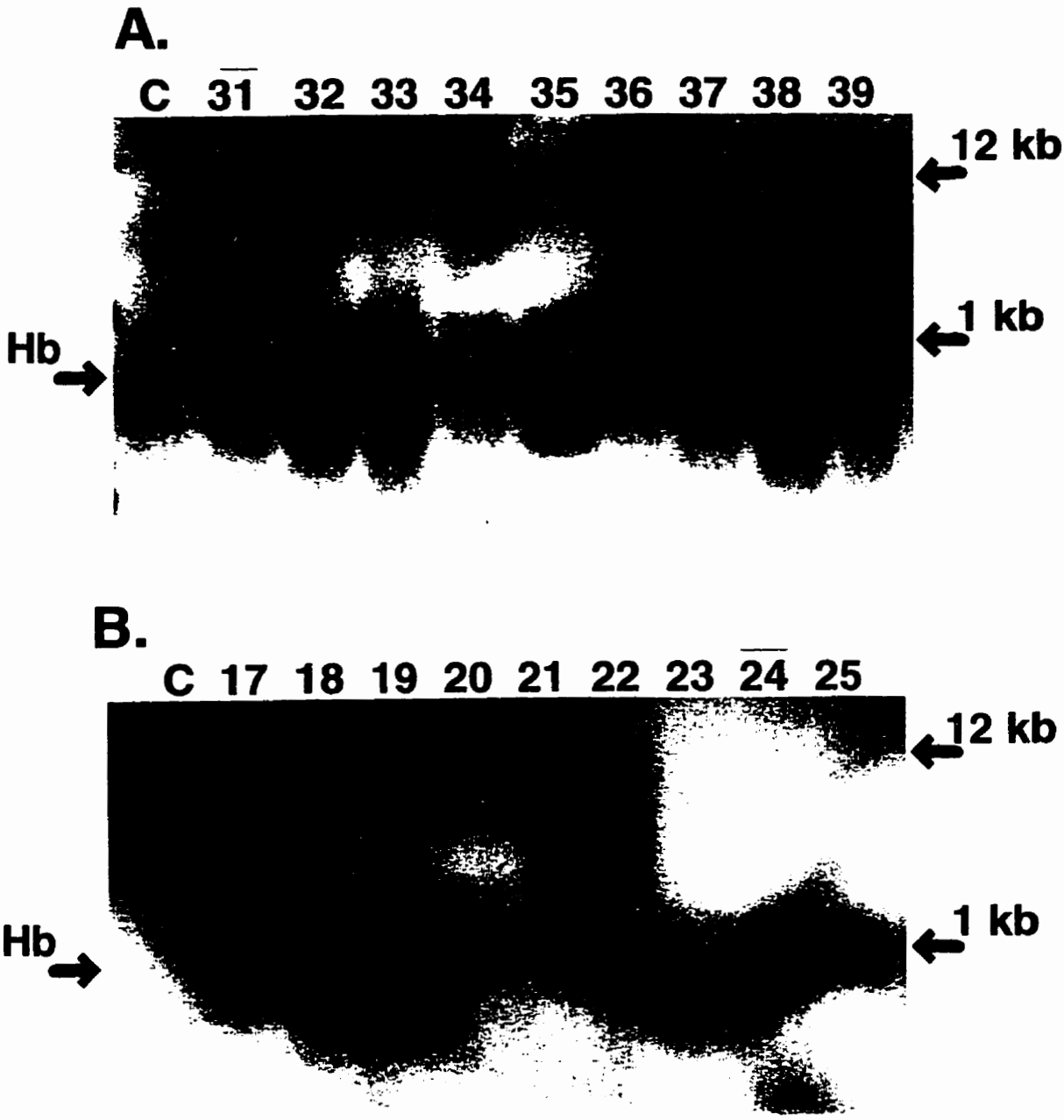


Figure 13. Southern blot analysis of selected maize colonies.

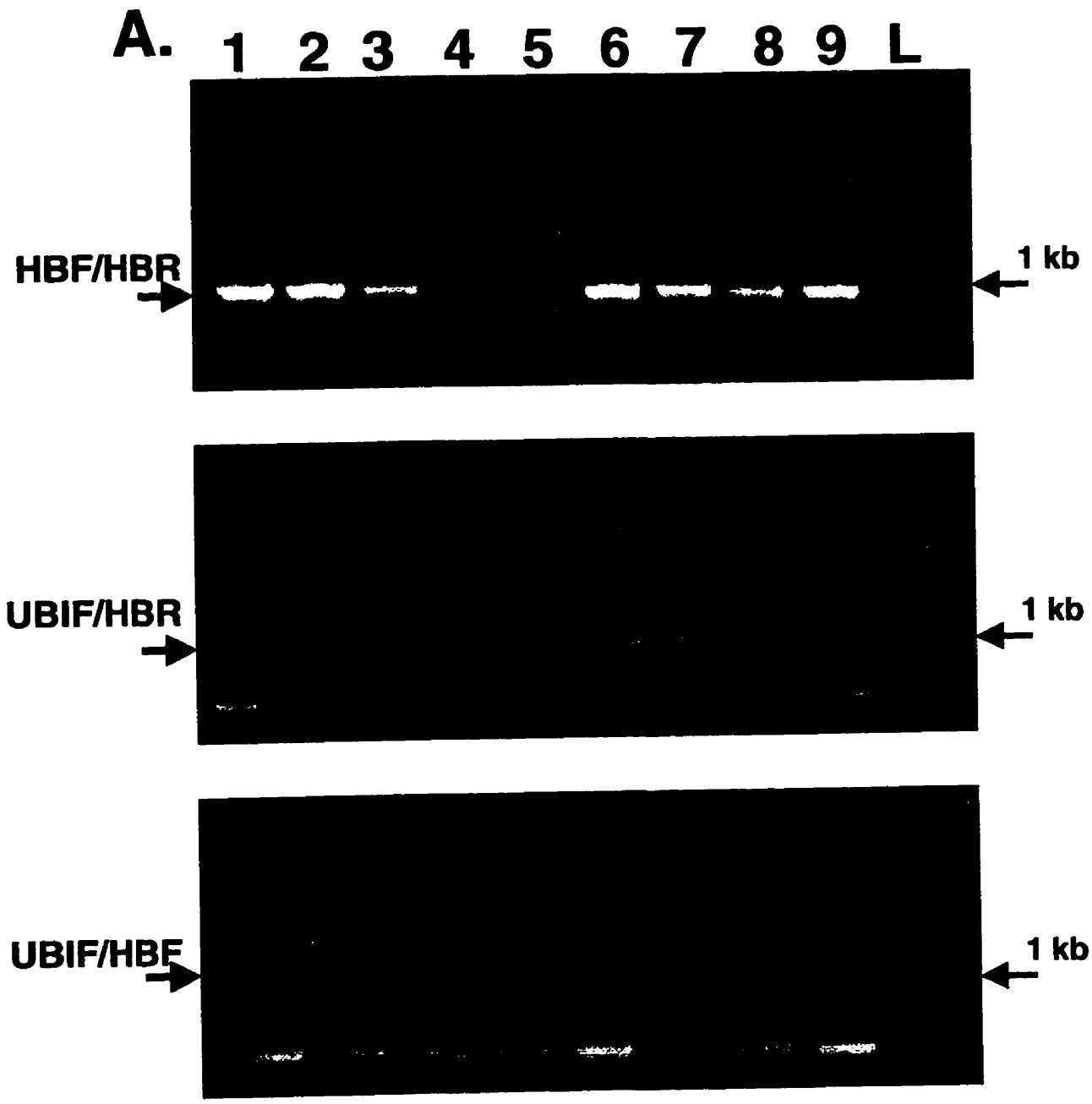
20 μg of genomic DNA, isolated from the glufosinate ammonium-selected maize cells, was digested with *PstI* that liberates 970 bp Hb cDNA from transformation constructs, and loaded into each lane. Hybridization was at 65°C, 6 x SSC, with barley Hb cDNA probe labelled with digoxigenin in a PCR reaction. Final washes were at 65°C, 0.1 x SSC. Detection was by chemiluminescence with 4 hours of film exposure. DNA of the untreated BMS cells was loaded into lanes marked C. Numbers on top indicate working names of selected colonies. Colonies 31 and 24 later became cell lines HB^+ and HB^- respectively. Sample blot of pAS1 transformed colonies is shown in panel A, and pAS2 transformed colonies in panel B.

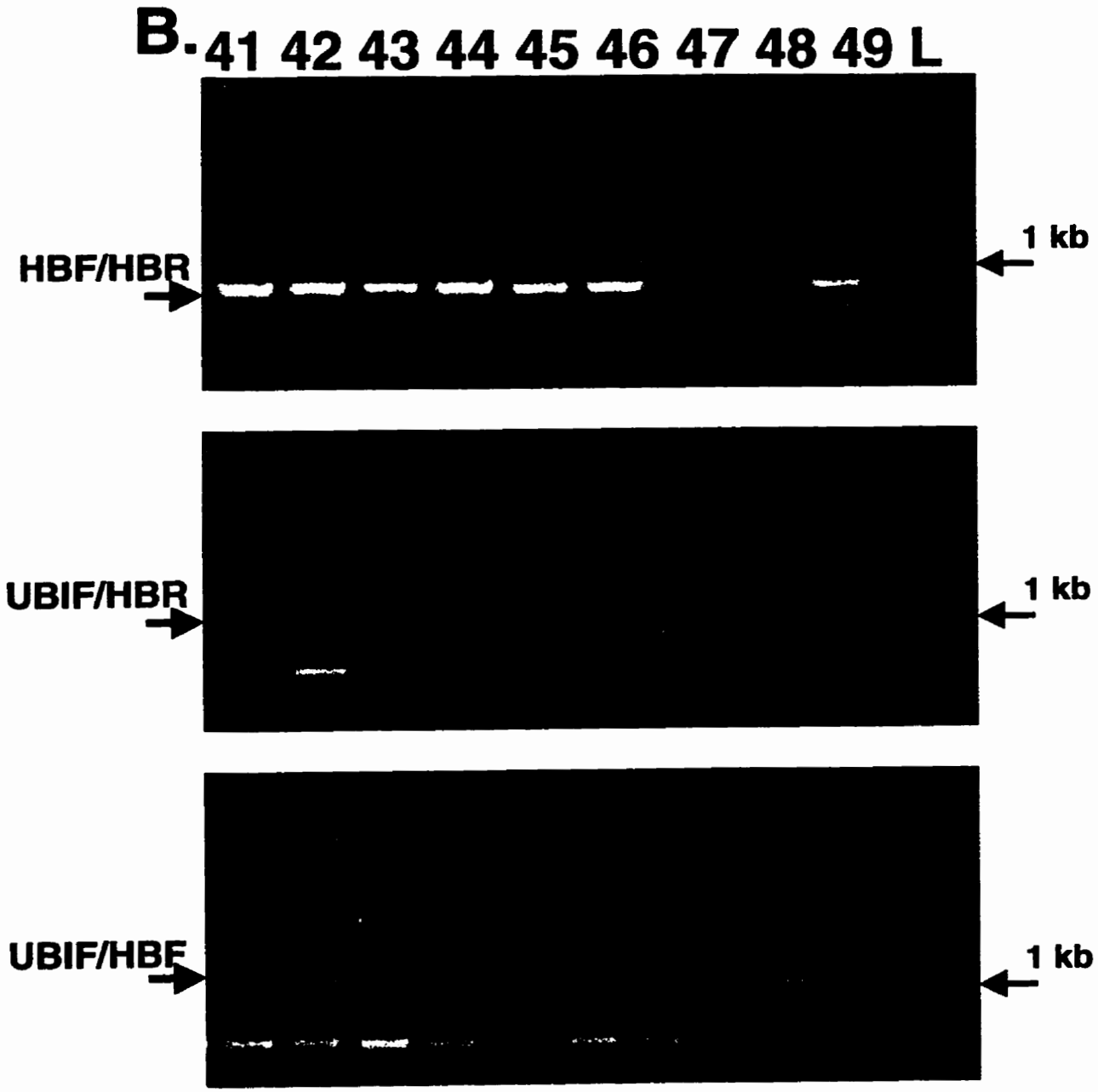

A.
C 31 32 33 34 35 36 37 38 39
12 kb
1 kb
Hb
B.
C 17 18 19 20 21 22 23 24 25
12 kb
1 kb
Hb

Out of 114 selected maize cell colonies only 62 contained full barley Hb cDNA (Table 2). Twenty four of these colonies were transformed with pAS1 (Hb sense construct), and 38 with pAS2 (Hb antisense construct) (Table 2). Examples of Southern blots of DNA from both Hb sense and antisense transformed colonies are presented in Figure 13. The lack of detection of the native maize Hb gene (8 kb hybridization band), in most lanes, may suggest some differences in nucleotide sequence between the maize and barley Hb genes or a presence of the transgene Hb gene in multiple copies.

Transformation of the BMS maize cells was confirmed with PCR analysis of their genomic DNA. PCR amplifications of genomic DNA with primers HBF and HBR, as described above for the construction of plasmid vectors, produced the expected 870 bp fragment from all cell colonies previously identified by Southern blot analysis. DNA from the untransformed BMS cells used as a template for PCR with HBF and HBR primers did not produce the 870 bp fragment (Fig. 14). This is expected since even if these two barley specific Hb cDNA primers hybridized to the native maize haemoglobin gene, the product of PCR amplification would be longer due to the presence of introns (Guy *et al.*, 1997).

Figure 14. PCR determination of Hb cDNA orientation in transformed cells. Genomic DNA isolated from transformed maize cells was used as a template in PCR reactions as described in fig. 8. The pAS1 (sense) transformants are shown in panel A, and the pAS2 (antisense) in panel B. Primer combinations and expected product sizes are indicated with arrows on the left. DNA size standards are marked L. Numbers on top indicate working names of selected cell lines. Cell lines 6 and 42 were later named HB^+ and HB^- respectively.


A.
1 2 3 4 5 6 7 8 9 L
HBF/HBR
1 kb
UBIF/HBR
1 kb
UBIF/HBF
1 kb


B.
41 42 43 44 45 46 47 48 49 L
HBF/HBR
1 kb
UBIF/HBR
1 kb
UBIF/HBE
1 kb

It is possible that a contamination or confusion of plasmid DNA samples prepared for transformation experiments, as well as DNA recombination after delivery, could result in other than expected orientation of the introduced haemoglobin genes. Genomic DNAs of all transformed BMS cells were, therefore, analysed for the orientation of introduced barley Hb cDNA by PCR with the UBIF/HBR and UBIF/HBF primer pairs. An example of such analysis is presented in Figure 14. All of the 24 pAS1 and 38 pAS2 transformed cell lines contained the barley Hb cDNA in proper sense or antisense orientation (Table 2). The correct PCR products were shown using the pAS1 and pAS2 transformation vectors as templates (Fig. 8). The presence of correct PCR amplification products, in reactions utilizing the UBIF primer, indicate that at least a part of the Ubi1 promoter was also present in the transformed cell lines.

Expression of haemoglobin in the transformed maize BMS cell lines

Transformation of maize BMS cells with pAS1 and pAS2 plasmid constructs was performed to alter expression of haemoglobin in these cells. The expression of haemoglobin in the transformed cell lines was, therefore, analysed by immunoblot detection with antibody raised against recombinant barley haemoglobin (Duff *et al.*, 1997). All of the tested pAS1 (sense) transformed cell lines expressed haemoglobin at levels significantly higher than the wild type (BMS), as assayed by the Western blot analysis (Table.3).

Table 3. ***Effectiveness of sense and antisense transformation***

Vector	Number of cell lines tested	Number of cell lines with altered Hb expression
pAS1	24	24 (increased)
pAS2	30	1 (decreased)

Hb expression was assayed with protein immunoblotting.

Transformation of a plant with a gene homologous to any gene that is a natural component of its genome, often results in an inactivation of both the transgene and the native gene, a phenomenon called cosuppression (Finnegan and McElroy, 1994). In the above described transformation of maize cells with barley haemoglobin gene, however, no cosuppression was observed (Table 3). Amongst 30 pAS2 (antisense) transformed cell lines only one revealed a detectable suppression of haemoglobin expression (Table 3). The observed lack of cosuppression amongst the sense transformants and the apparent low effectiveness of the antisense suppression of maize Hb gene may suggest a low sequence homology between the barley and maize genes, since both antisense and cosuppression are homology dependent. Alternatively, the cell survival under culture conditions may depend on the presence of haemoglobin, thus a strong suppression of the Hb gene leads to cell death during the selection process.

HB^+ and HB^- maize cell lines

One pAS1 transformed cell line, with the highest expression of haemoglobin at the protein level, and the only recovered cell line with decreased expression of haemoglobin were selected for further experiments. These selected lines were named HB^+ (sense) and HB^- (antisense). No significant changes in the general protein expression pattern were observed between the transgenic and wild type cell lines, as visualized by SDS-PAGE of total soluble protein (Fig. 15). There is no visible difference, between the three cell lines, in intensity of 18.5 kDa bands, the size of barley and maize haemoglobin (Duff *et al.*, 1997; Silva, 1997).

Expression of haemoglobin in HB^+ and HB^- cells

To determine haemoglobin expression levels in these transgenic cell lines, total soluble protein was extracted from cells grown under normal culture conditions (see materials and methods), and its haemoglobin content assayed by immunoblots with anti-haemoglobin (recombinant) polyclonal IgGs. The western blot bands were compared to a standard curve of known amounts of the barley recombinant Hb. The HB^+ cells express haemoglobin at the level of about 1.2 % of the total soluble protein, that is ten fold higher than that of the wild type BMS cells (Fig.16). Haemoglobin expression in the HB^- cells has been decreased to about 0.015% of the total soluble protein, a level ten fold lower than that of the wild type BMS cells (Fig.16).

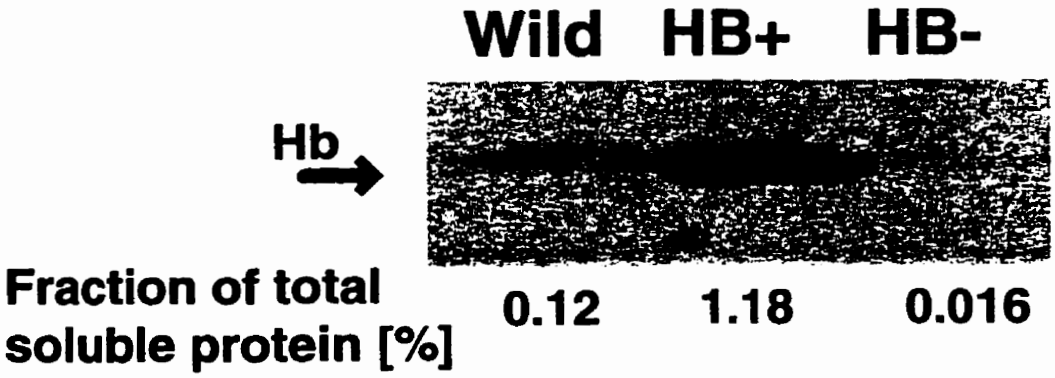
Figure 15. Electropherogram of total buffer extractable protein of maize HB+, HB- and wild type BMS cells.

60 μg of total soluble protein were loaded into each lane. 2μg of purified recombinant barley Hb (18.5 kDa) were loaded into lane marked r HB.


HB+
Wild
HB-
r Hb
Hb

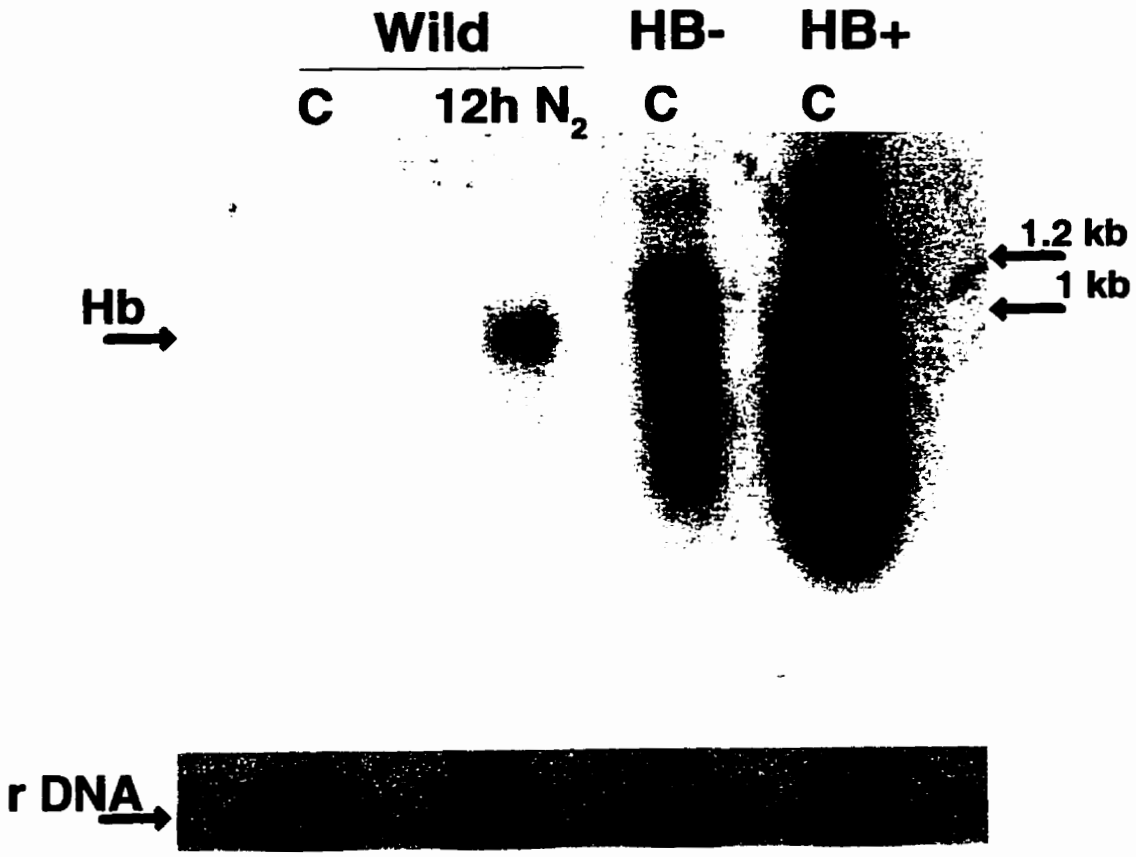
Figure 16. Protein immunoblot analysis of haemoglobin expression in HB^+, HB^- and wild type BMS cells.

100 μg of total soluble protein isolated from normally grown HB^+, HB^- and wild type BMS cells were loaded into each lane. Polyclonal antibody, raised and titrated against barley recombinant haemoglobin (Duff *et al.*, 1997) was used for immunodetection. Numbers below the blot indicate haemoglobin content of protein extracts expressed as the percentage of the total soluble protein. Values were obtained by scanning band intensity of four blots and comparison to the standard curve of known amounts of recombinant barley Hb.


Wild
HB+
HB-
Hb
Fraction of total soluble protein [%]
0.12
1.18
0.016

The expression of haemoglobin at the protein level corresponds to the amount of Hb mRNA detected in these cells. Northern blot analysis of RNA isolated from the HB^+, HB^- and the wild type BMS cells shows a massive increase in the levels of HB^+ cells compared to the wild type (Fig. 17). The probe, prepared from double-stranded barley Hb cDNA, detects both sense and antisense RNA. However, due to the construction of plasmid vectors, the antisense RNA is about 200 bp longer than the sense one. The full length barley haemoglobin cDNA, used in the construction of plasmid vectors, contains the transcription stop and polyadenylation signals (Taylor *et al.*, 1994). Transcription from the sense construct (pAS1), therefore, stops at the end of the cDNA sequence, producing transcript of about 940 bp. When the cDNA is placed in the antisense orientation (pAS2), the transcription stop and polyadenylation signals are not present and transcription extends into the NOS terminator (see Fig. 7). This results in a transcript of about 1140 bp. Figure 17 shows abundance of the antisense transcript and lack of detectable sense Hb mRNA in the HB^- cells. The amount of the antisense Hb mRNA in HB^- cells is considerably lower than the amount of the sense transcript in HB^+. This may be a result of higher transgene copy number in HB^+.

Figure 17. Northern blot analysis of haemoglobin expression in transgenic maize cells. 10 μg of total RNA were loaded into each lane. RNAs of normally cultured cells are marked C. RNA size markers are indicated on the right. Barley Hb cDNA probe was ^{32}P-labelled by random hexanucleotide priming. After autoradiography membrane was stripped and reprobed with ribosomal DNA probe (rDNA) to ensure equal loading. Hybridizations were at 65°C, 6 x SSC. Final wash was at 65°C, 0.1 x SSC. Autoradiography was for 16 hours.


Wild
HB-
HB+
C
12h N2
C
C
1.2 kb
1 kb
Hb
r DNA

Expression of haemoglobin in HB^+ and HB^- cells under stress

Transcriptional activity of maize ubiquitin Ubi1 promoter, although constitutive and high under normal growth conditions, was reported to be induced by various environmental stresses in certain cell types (Takimoto *et al.*, 1994).

To determine whether environmental stress has any effect on the expression of haemoglobin in transgenic maize cells, the cultures were exposed to heat shock (4 hrs at 38°C) and anoxia (N_2 atmosphere) for 24 hours. The haemoglobin content of protein extract was analysed by protein immunoblotting, as described above. No detectable change in haemoglobin expression was observed in the transformed HB^+ and HB^- cells (Fig. 18). A slight increase in haemoglobin was noted in the HB^+ cells subjected to heat shock. No detectable changes in haemoglobin expression, at the protein level, were observed after anoxic treatment of any of the three cell lines (Fig. 18).

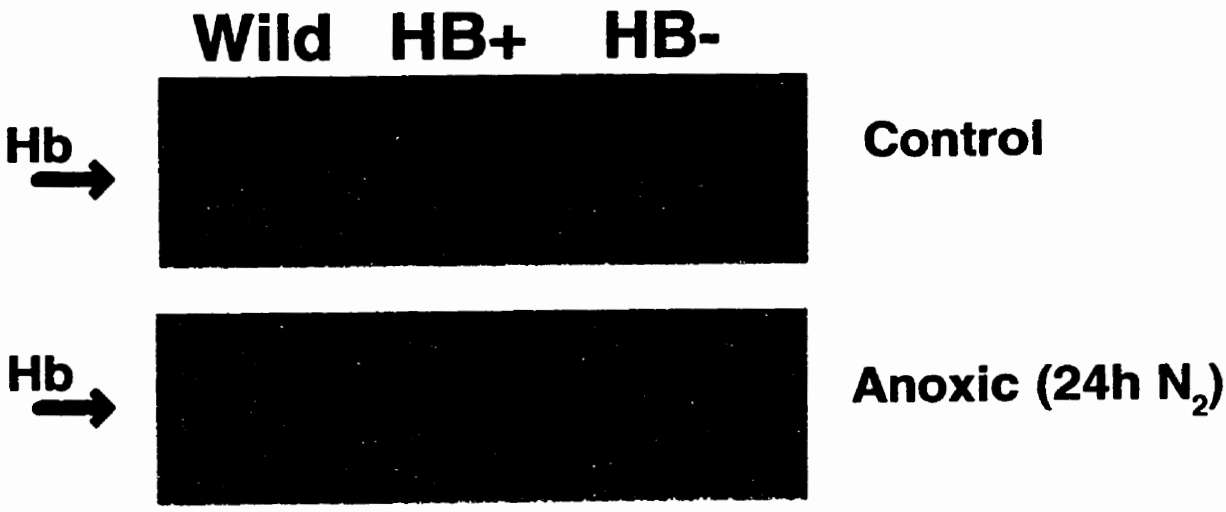
Purification of haemoglobin from maize cells

The above described experiments show that the expression of sense and antisense Hb RNA altered the amount of Hb protein in transgenic maize cells. It should be noted, however, that the functional haemoglobin consists of the Hb polypeptide and noncovalently bound prosthetic group, haem. Physiological effects of transformation, if any, may be only expected if the transgene haemoglobin is capable of haem binding, and that the process is not limited, in maize cells, by availability of haem or by any other factors.

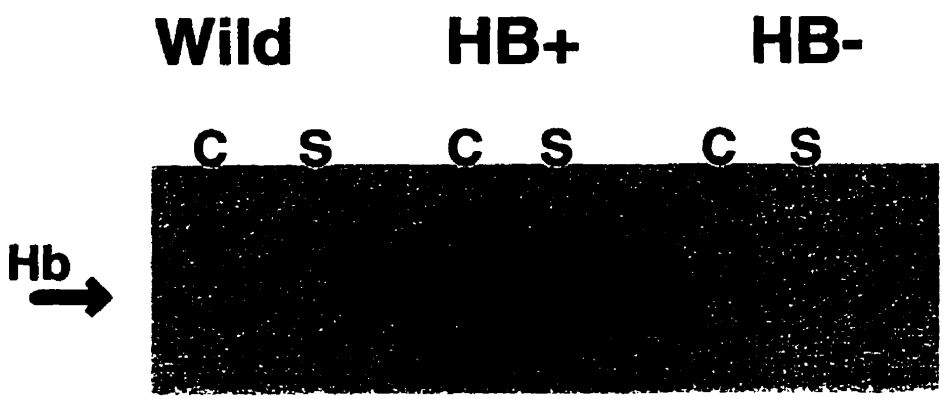
Figure 18. Protein immunoblot analysis of haemoglobin expression in transgenic maize cells under stress.

60 μg of total soluble protein, isolated from normally grown and stressed by anoxia (panel A) and heat shock (panel B) cells, was loaded into each lane.

A.



B.



C - Grown at 25°C

S - Heat stressed (4h at 37°C)

Since protein immunoblotting, used to detect altered levels of Hb polypeptide, does not distinguish between the haem-bound and free Hb polypeptide, haemoglobin was purified from the transformed HB^+ and wild type BMS cells. Purifications were performed with the use of FPLC (Pharmacia) chromatography system (see materials and methods).

The purification of recombinant haemoglobin from HB^+ cells is shown in Table 4. Haemoglobin was purified 75 fold to a final purity of over 89%, as assessed by A_{412} measurements, with the yield of 4%. Purified haemoglobin was over 90% pure as assessed by SDS-PAGE (Fig.19). About 1 mg of haemoglobin was recovered throughout the purification process. Western blot analysis of the purified protein (Fig.19) confirms that the recovered protein is in fact haemoglobin.

The same purification procedure of the natural maize haemoglobin from the wild type BMS cells, starting with the same amount (1500 mg) of the crude protein extract was carried only to the step of Phenyl Sepharose chromatography (Table 5). Maize haemoglobin was purified 6 fold to a purity of about 0.6%, with the yield of 5.5%. The 18.5 kD band is barely visible in the SDS-PAGE gel (Fig 20). Due to the low yields of haemoglobin large fractions had to be collected, and therefore, purification was not effective. Western blot analysis of the purification product (Fig. 20) shows, that the sample in fact contained maize haemoglobin.

Table 4. ***Purification of haemoglobin from HB$^+$ cells***

Fraction	Protein	Hb	Hb	F. P.[b]	Yield
	mg	mg	%		%
Crude extract	1560	18.7[c]	1.2	1	100
40-80 % Ammonium sulphate precipitation	702	13.4[a]	1.9	1.6	72
Q-Sepharose chromatography	63	7.1[a]	11.2	9.3	38
Phenyl - Sepharose	10	4.6[a]	46	38.3	24
Mono-Q FPLC	5	3.6[a]	72	60	19
Phenyl-Superose FPLC	1	0.9[a]	90	75	4

[a] Amount of Hb determined by A_{412} measurements and a standard curve

[b] Fold purification

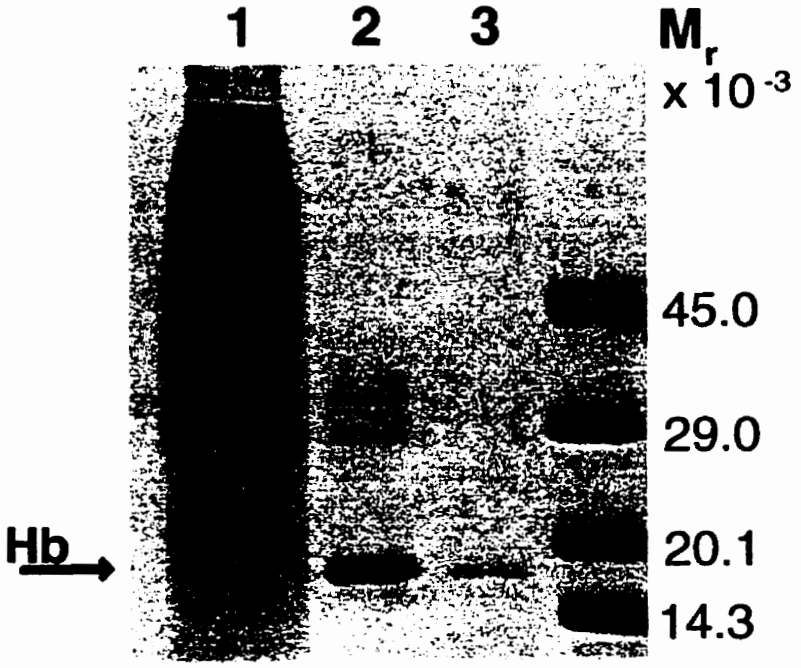
[c] Amount of Hb assumed from Western blot scans of HB$^+$ cells

Figure 19. Purification of haemoglobin from HB^+ cells.

Panel A. 60 μg of total soluble protein isolated from HB^+ transgenic maize cells were loaded into lane 1. 2 μg of haemoglobin purified from HB+ cells were loaded into lane 2. 100 ng of barley recombinant haemoglobin were loaded into lane 3. Protein size standards are in far right lane, with markers indicated on the right.

Panel B. Western blot analysis of protein samples of lanes 1, 2, and 3. Detection was with anti- barley recombinant Hb antibody.

A.



B.

Table 5. ***Purification of maize haemoglobin from wild type BMS cells***

Fraction	Protein	Hb	Hb	F. P.[b]	Yield
	mg	mg	%		%
Crude extract	1600	1.8[c]	0.1	1	100
40-80 % Ammonium sulphate precipitation	783	1.4[a]	0.18	1.8	77
Q-Sepharose chromatography	150	0.8[a]	0.4	4	44
Phenyl - Sepharose	15	0.1[a]	0.6	6	5.5

[a] Amount of Hb determined by A_{412} measurements and a standard curve

[b] Fold purification

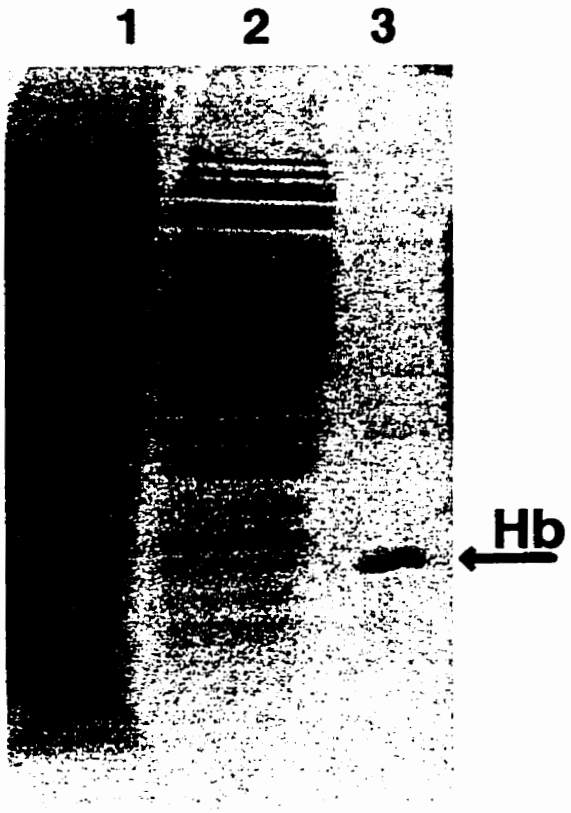
[c] Amount of Hb assumed from Western blot scans of HB^- cells

Figure 20. Purification of haemoglobin from wild type BMS cells.

Panel A. 60 μg of total soluble protein isolated from BMS maize cells were loaded into lane 1, and sample after purification was loaded into lane 2. Recombinant barley Hb standard is in lane 3.

Panel B. Western blot analysis of samples shown in panel A. Detection was with anti-barley recombinant Hb antibody.

A.



B.

During both purification procedures, samples were collected on the basis of their absorption at 412 nm and Western blotting. The absorption at 412 nm (Soret peak) is characteristic of haemoproteins and together with SDS gels and protein immunoblots (Fig. 19), show that the isolated protein is a fully assembled haemoglobin. A comparison of tables 4 and 5 shows clearly that the HB^+ cells contain higher amounts of haemoglobin than the wild type cells .

Barley haemoglobin purified from the HB+ cells is red in colour, as expected of a haemoprotein (Fig. 21). Spectral analysis of the oxygenated and deoxygenated ferrous haemoglobin isolated from HB^+ cells is presented in table 6. The oxygenated haemoglobin shows the Soret peak at 412 nm and two peaks in the visible region at 540 and 567 nm. The Soret peak of the deoxygenated (by excess of dithionate) haemoglobin shifts to 424 nm with two major peaks at 528 and 562 nm, and two minor peaks at 534 and 554 nm, in the visible region. These absorption peaks, as well as the extinction coefficients (Table 6) are identical with those of recombinant barley haemoglobin (Duff *et al.*, 1997). These spectral properties of barley haemoglobin, expressed in the transgenic maize cells are characteristic of low spin 6-C haemoproteins (Duff *et al.*, 1997).

Figure 21. Photograph of haemoglobin sample purified from HB^+ cells.

SC. 3
Park
N.Y. 25

Table 6. ***Spectral properties of haemoglobin from HB⁺ cells***

Derivative	λ_{max}	ϵ_m^M
Ferrous Hb	424	190
HbO_2	412	149
	540	17.2
	576	17.2

Summary

A nonsymbiotic haemoglobin gene is present and expressed in maize (BMS) suspension cultured cells. Similar to barley aleurone and root tissues (Taylor *et al.*, 1994), and maize roots (Silva, 1997), the haemoglobin gene is hypoxia inducible in maize suspension cells.

Transformation vectors were prepared with barley Hb cDNA, in both sense and antisense orientations, under the control of a strong, constitutive promoter of the maize ubiquitin (Ubi1) gene. The silicon carbide fibres-mediated genetic transformation of maize (BMS) cells yielded 24 cell lines with the sense barley Hb construct incorporated in their genome, and 38 lines transformed with the antisense Hb construct.

While all sense transformants expressed increased levels of Hb polypeptide, only one out of 38 antisense lines showed a marked decrease in the level of haemoglobin expression. The antisense line (HB^-) expresses haemoglobin at the level of 0.01% of the total soluble protein, ten fold lower than that of the wild type BMS cells. The haemoglobin content of the HB^+ cells, a line selected from the sense transformants, is about 10 times higher than that of the wild type cells.

The expression of haemoglobin in the transgenic maize cells is not affected by low oxygen availability, and in the case of the sense line (HB^+) can be additionally increased by a heat shock stress. Purification of haemoglobin from both wild type and HB^+ cells shows that the transgene barley haemoglobin is properly assembled and haem-bound in maize cells. Furthermore, the spectral characteristics of oxygenated and deoxygenated ferrous haemoglobin purified from the HB^+ cells are identical with those of the recombinant barley

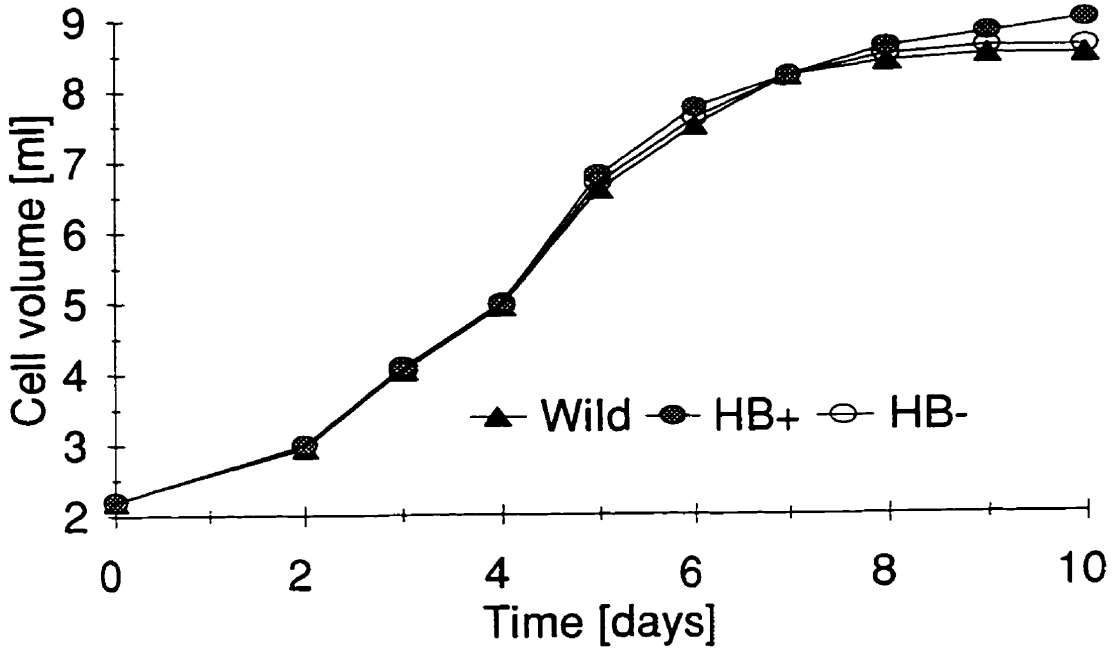
haemoglobin, indicating that the protein is functional in the transgenic maize cells.

The HB^{+}, HB^{-} an the wild type BMS cells are genetically different only with respect to the introduced gene constructs and constitute a system of three expression levels of nonsymbiotic haemoglobin. These cell lines have been used in experiments aimed at demonstrating of the effects of haemoglobin expression in maize cells.

Expression of haemoglobin has no effect on the cell culture growth under normal air conditions

The transformed cell lines did not differ from the wild type BMS cells with respect to the general protein expression pattern, as analysed by SDS-PAGE gels (Fig.15). To investigate whether the presence of haemoglobin has any major effect on the general physiology of maize cells, the growth of the HB^+, HB^-, and the wild type BMS cell cultures was measured over a period of 10 days. Normally, the cells were subcultured every 7 days (see materials and methods). All three cell lines showed a typical sigmoidal growth curves (Fig. 22), with the most intensive growth between the third and the sixth day of culture. After the sixth day of culture, the cell growth rates begin to decrease and, in the case of HB^- and the wild type, the culture growth practically ceased by the eight day of culture. The HB^+ cell cultures show a slight growth after the eighth day, the difference, however, is negligible (Fig. 22). Over the course of experiments the volume of sedimented cells in the three cell lines, had increased by over four fold. These growth curves indicate that the cultures are most active between the third and the sixth day of culture and, therefore, future analyses should be conducted at this stage of growth. When the cultures are placed in an atmosphere of pure nitrogen, cell growth arrests (data not shown).

Figure 22. Growth of transgenic and wild type BMS maize cell cultures. Cultures were grown at 25°C in the atmosphere of normal air. Cell volume was measured after sedimentation in 25 ml graduated pipette. Maximum standard error of four repeats was less than 5%.


Cell volume [ml]
9
8
7
6
5
4
3
2
0
2
4
6
8
10
Time [days]
Wild
HB+
HB-

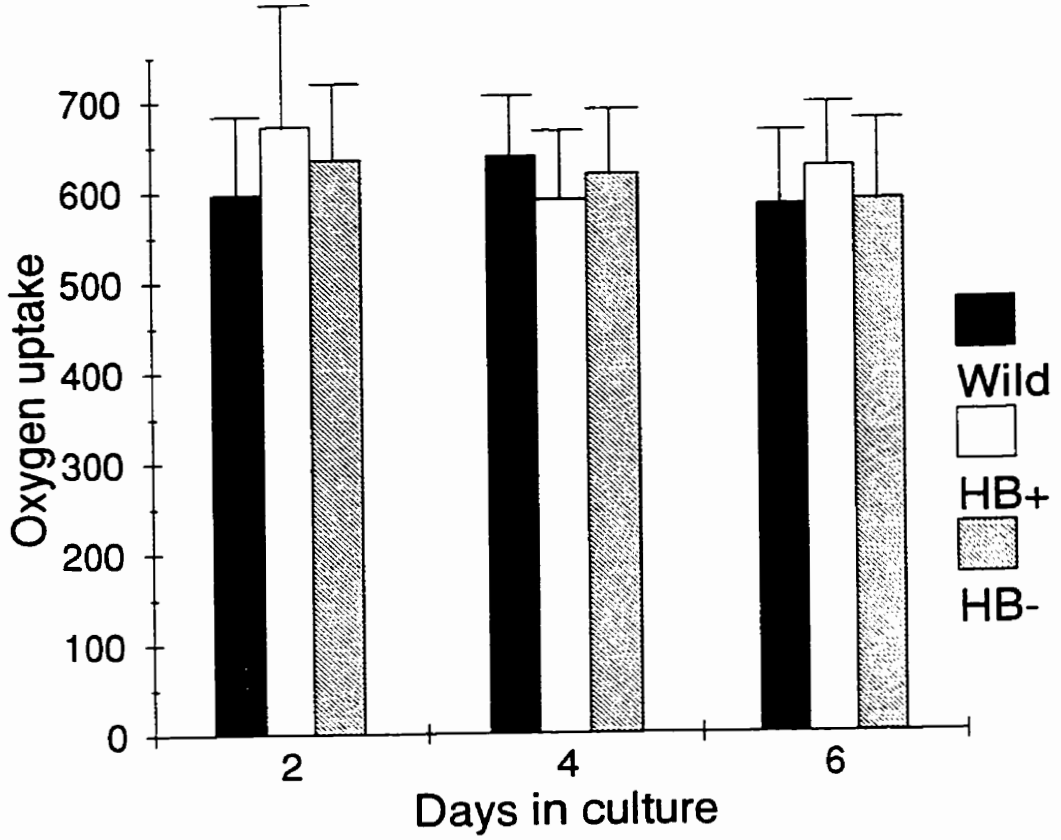
Oxygen uptake by maize cells grown under normal air conditions is not affected by haemoglobin

Haemoglobins have been shown to function, in various systems, as oxygen transporters or facilitators of its diffusion (for review see: Giardina *et al.*, 1995; Appleby, 1992; Wittenberg and Wittenberg, 1990). To investigate whether the level of haemoglobin expression may have an effect on oxygen uptake and utilization by maize cells, the oxygen uptake by the three cell lines was measured at three different time points of the culture cycle (Fig. 23). Oxygen uptake measurements were performed on cells grown under normal air conditions and, during these measurements, the cells were placed in fully aerated medium. The value of 260 μM was taken for oxygen concentration in the aerated medium at 25°C. The cells were sampled at the second, fourth, and the sixth day of culture, representing the very beginning, middle, and the end point of the intensive cell growth period (Fig. 22).

These measurements did not reveal any significant differences between the three cell lines at any of the time points tested. This may suggest that haemoglobin, at the levels found in the HB^+, HB^- and the wild type BMS cells, does not contribute to the uptake and utilization of oxygen under the conditions of normal air atmosphere.

Figure 23. Oxygen uptake by maize cells under normal air conditions.

Oxygen uptake was measured polarographically with an O_2 electrode. Measurements were performed 2, 4 and 6 days after subculture. Line bars indicate standard error of three experiments. Oxygen uptake is expresses as nmol $O_2 \cdot min^{-1} \cdot 1g^{-1}$ fw cells.


Oxygen uptake
700
600
500
400
300
200
100
0
2
4
6
Days in culture
Wild
HB+
HB-

Expression of nonsymbiotic haemoglobin affects fermentative metabolism in maize cells

An induction of a nonsymbiotic haemoglobin has been observed in barley aleurone and root tissues (Taylor *et al.*, 1994), barley leaves (Nie, 1997), maize roots (Silva, 1997), and roots of *Arabidopsis thaliana* (Trevaskis *et al.*, 1997), subjected to low oxygen environments. Based on the kinetics of this induction it has been proposed by Taylor *et al.* (1994), that the expression of the nonsymbiotic haemoglobin may be an integral part of the plants response to limiting oxygen stress. In situations where oxygen availability is not sufficient to sustain oxidative phosphorylation, fermentative metabolism becomes the main source of energy production in plants (Ricard *et al.*, 1994; Kennedy *et al.*, 1992).

To investigate whether the expression of haemoglobin influences fermentative metabolism in maize cells, the activities of the main fermentative enzymes, alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH), were assayed along the time course of hypoxic exposure.

The base level of ADH activity under normal air conditions, is the same for the HB^+, HB^-, and the wild type BMS cells, and measures 0.07 U [U = μmol NAD $\cdot$ min^{-1} $\cdot$ mg^{-1} protein] (Fig.24). With exposure to an N_2 atmosphere, ADH activity in the wild type cells increases steadily, reaching 0.45 U (over 6 fold increase) by 24 hours of exposure (Fig. 24). The ADH activity in the HB- cells, after a 6 hour lag period, follows that of the wild type cells. The constitutively expressing haemoglobin HB^+ cells show a lower degree of ADH induction under the atmosphere of nitrogen. After 6 hours exposure to N_2 atmosphere, the ADH activity in the HB^+ cells increases, but remains at 30 to 40 % lower

than that of the wild type and HB^- cells (Fig. 24).

Similar to ADH, the activity of lactate dehydrogenase (LDH) under normal atmospheric conditions, is the same for all of the three cell lines (Fig. 25). A slight induction in LDH activity was observed at 4 hours of hypoxic exposure, with the HB^- showing the highest level of induction. It should be noted, however, that the LDH activity was 10 to 40 times lower than that of ADH (Fig. 25), indicating that lactic fermentation does not contribute significantly to anaerobic energy production.

The observed lower ADH activity in the HB^+ cells, suggests a lower activity of fermentative metabolism, compared to the HB^- and the wild type BMS cells,. This could be explained by one of the following speculative scenarios. First, the HB^+ were, simply, less anaerobic due to the oxygen available to their mitochondrial respiration through haemoglobin-mediated diffusion or storage. Second, haemoglobin functions to reoxidise the reductant (NADH) in a pathway other than the observed fermentative route. Third, the presence of haemoglobin enables a mean of energy production other than via glycolysis or oxidative phosphorylation. It is possible, however, that a lower ADH activity in the HB^+ cells resulted from an increased cell death rate. Experiments presented below were aimed at better understanding of the nature of this phenomenon.

Figure 24. Activity of alcohol dehydrogenase of transgenic and wild type cells under hypoxia.

Cell cultures were placed in the atmosphere of N2 for 6, 12 and 24 hours. ADH activity was measured as described in materials and methods. ADH activity is expressed as μmol ·· min-1 ··mg-1 protein [U] ± SE (n=3).

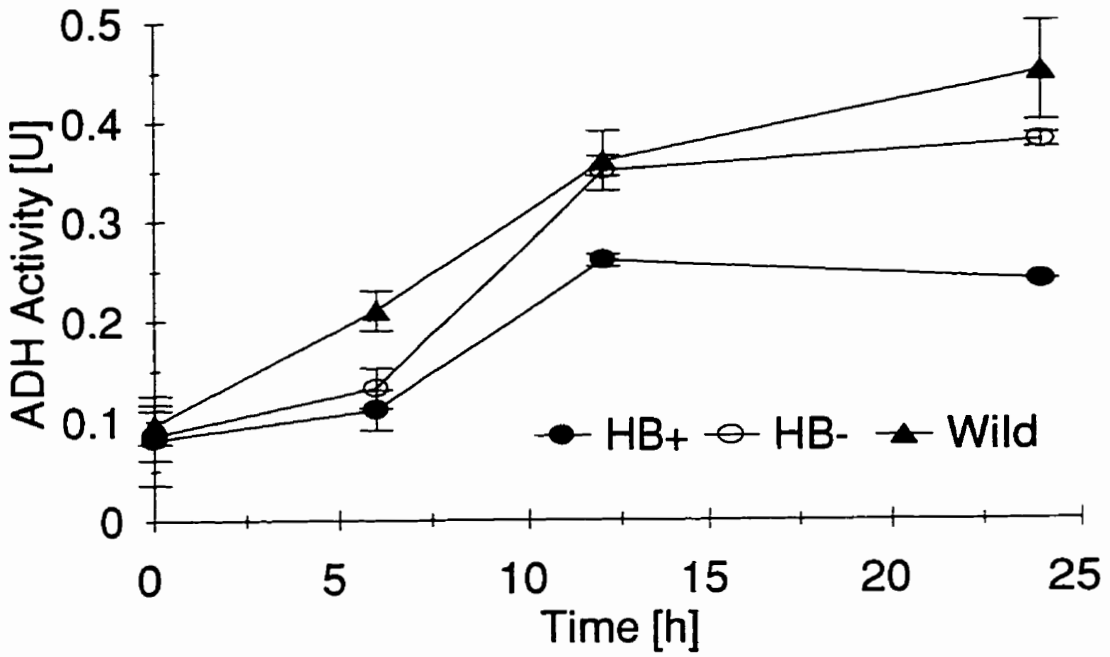
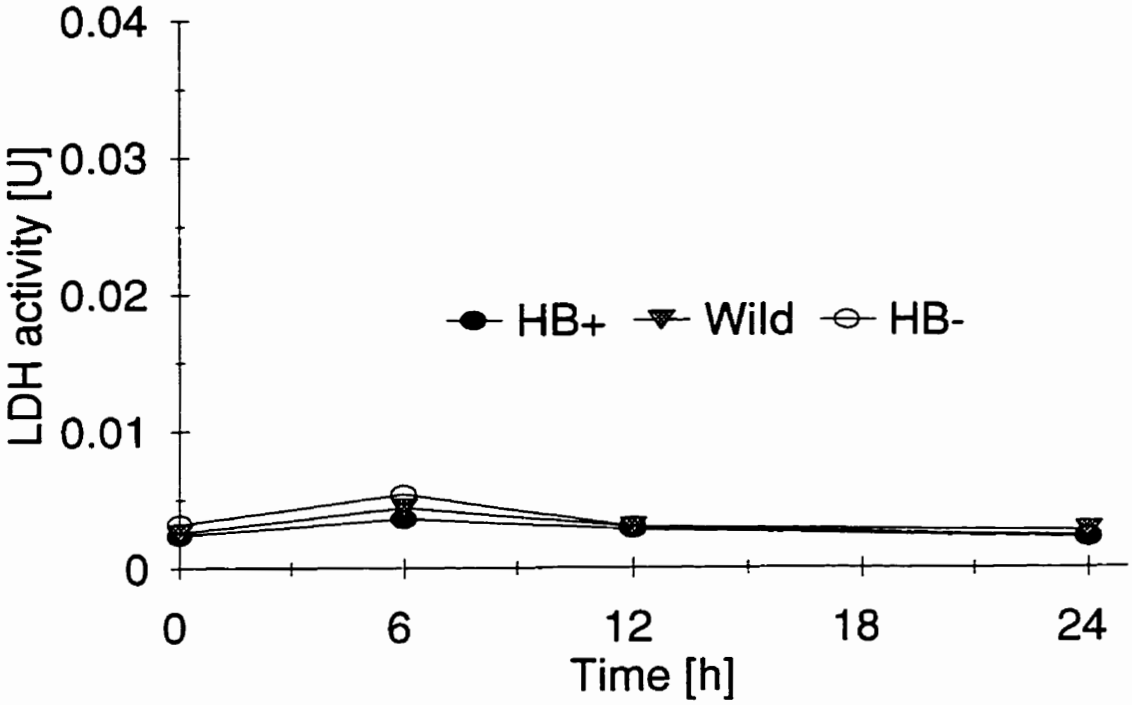

0.5
0.4
0.3
0.2
0.1
0
ADH Activity [U]
HB+
HB-
Wild
0
5
10
15
20
25
Time [h]

Figure 25. Activity of lactate dehydrogenase of transgenic and wild type cells under hypoxia.

Cell cultures were placed in the atmosphere of N2 for 6, 12 and 24 hours. LDH activity was measured as described in materials and methods. LDH activity is expressed as μmol - min-1 - mg-1 protein [U]. Maximum SE (n=3) was less than 15%


0.04
0.03
0.02
0.01
0
LDH activity [U]
HB+
Wild
HB-
0
6
12
18
24
Time [h]

Energy status of maize cells under hypoxia is affected by their ability to synthesize nonsymbiotic haemoglobin

The observed differences, between the three cell lines, in ADH induction under low oxygen atmosphere (Fig. 24), raise questions concerning the energy status of these cells. In order to gain some further insight into the energy metabolism of the maize cells, total pools of ATP, ADP, and AMP were assayed in cells grown under normal air conditions and after 12 hours of exposure to an atmosphere of N_2. The differences in ADH activity between the three cell lines, were already visible after 12 hours of hypoxic stress (Fig. 24).

The results of the adenylate pools measurements are presented in Figure 26 and table 7. ATP constituted over 85% of the total adenylates pool in HB+ and wild type cells, and in all cases the AMP was either undetectable or close to zero. Therefore, only the ATP values are presented in a separate graph (Fig. 26). In the hypoxia-stressed HB$^-$cells ATP accounted for only 55% of the total adenylates (Fig. 26; Table 7). The ADP and AMP values were used to calculate the energy charge and the total adenylates (Table 7).

Under normal air conditions, the steady state ATP levels were the same in the three types of cells (Fig. 26). Similarly, no significant differences in energy charge or total adenylates were observed under these conditions (Table 7). After incubating the cells for a further 12 hours in an atmosphere of nitrogen, significant differences were noted in the ATP levels of the cell types.

Figure 26. ATP levels in transgenic and wild type BMS maize cells.

ATP was measured spectrophotometrically in samples of wild type (BMS), HB^+ and HB^- maize cells grown under normal atmospheric conditions, and after 12 hours of treatment with N_2, Antimycin A (0.2 mM) and N2 in combination with Antimycin A. SE (n=3) in all measurements was less than 4%.

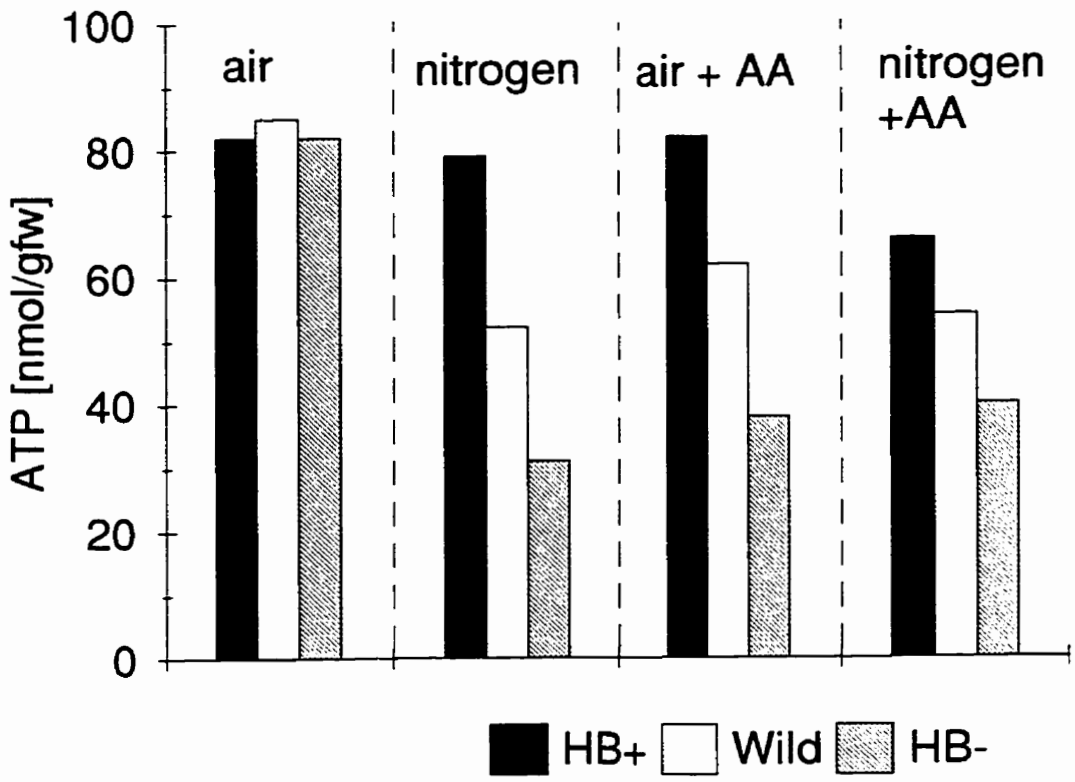

100
80
60
40
20
0
ATP [nmol/gfw]
air
nitrogen
air + AA
nitrogen
+AA
HB+
Wild
HB-

Table 7. ***Energy charge and total adenylates in maize cells before and after exposure to nitrogen atmosphere for 12 hours.***

Results are expressed as nmol per g fresh weight. Maximum SE (n=3) was less than 5%.

Cell line	Energy Charge		Total Adenylates (nmol per g fresh weight)	
	Air	Nitrogen	Air	Nitrogen
HB^+	0.93	0.93	96	92
Wild	0.94	0.93	94	61
HB^-	0.91	0.73	99	59

The level of ATP was highest in the HB^+ cells, being only marginally lower than under normal air conditions. ATP levels in the wild type (BMS) cells were 27% lower than HB^+ cells, while those in HB^- cells were 61% lower (Fig. 26).

Differences in energy charge and total adenylates were also observed in cells exposed to a nitrogen atmosphere (Table 7). Energy charge was similar in all three cell types, grown under normal atmospheric conditions, varying between 0.91 to 0.94. After 12 hours of exposure to a nitrogen atmosphere the HB^+ and the wild type cells maintained their energy charge at 0.93. HB^- cells, on the other hand, were unable to maintain energy charge, which decreased to 0.73 (Table 7).

Total adenylates remained at the same level in all three cell lines under normal atmospheric conditions. After 12 hours in a nitrogen atmosphere, the levels of total adenylates remained unchanged in the HB^+ cells. In the BMS and HB- cells, however, the total adenylate declined by about 35% (Table 7).

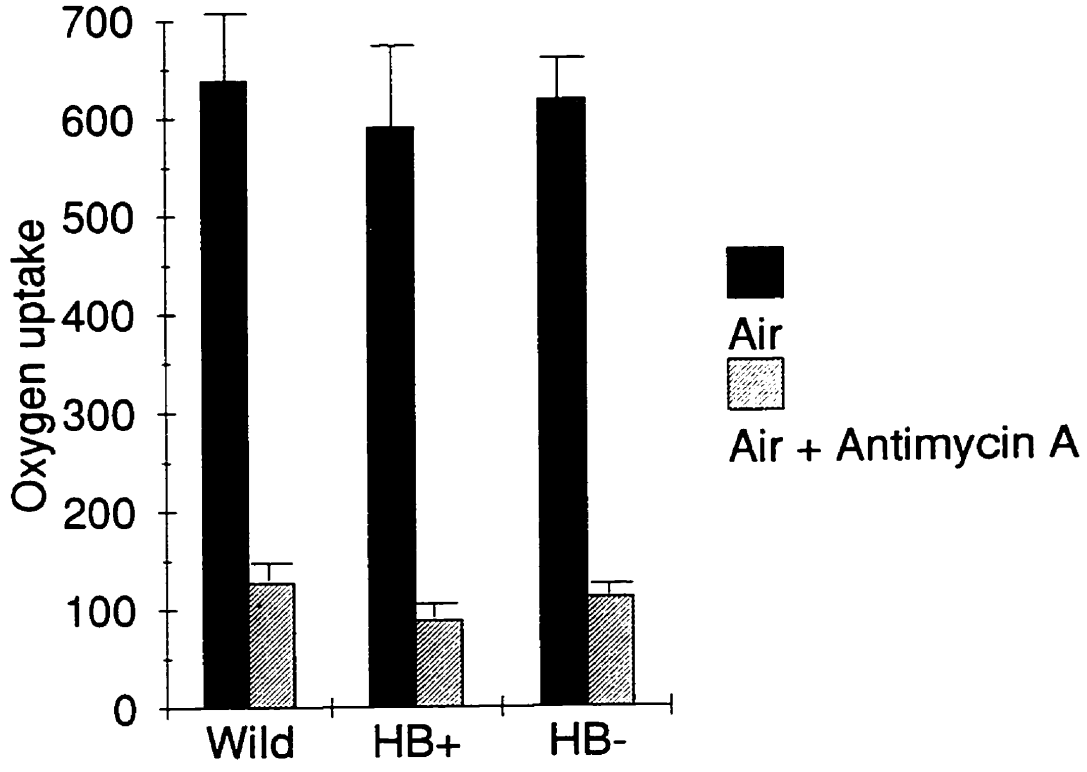
The above results link nonsymbiotic haemoglobin expression with the energy metabolism under low oxygen availability. The question as to what part of the cell metabolism is involved in this increased ability to maintain energy status in the presence of haemoglobin, remains to be answered.

Energy status of maize cells under hypoxia and mitochondrial electron transport

To examine the possibility that haemoglobin might provide oxygen to generate ATP via cytochrome-mediated respiratory process, ATP levels were measured in cells incubated with antimycin A (0.2 mM). Antimycin A blocks mitochondrial electron transport in the span from cytochrome b to c and has been shown to induce haemoglobin expression in barley aleurone layers (Nie and Hill, 1997). Antimycin A inhibited about 80% of the oxygen uptake, in all three cell types, within 30 min of treatment (Fig. 27). The initial inhibition of oxygen uptake, measured within 5 min after addition of antimycin A, reached between 30 to 40% in all three cell lines (data not shown). After 12 hours exposure to antimycin A in an air environment, ATP levels in the three cell lines were similar to those of the untreated cells after 12 hours in nitrogen atmosphere (Fig. 26). Upon placing the antimycin A treated cells in the atmosphere of nitrogen for 12 hours, a decrease in ATP levels was observed in the HB^+ and wild type cells, but consistent with the previous experiments, the levels of ATP decreased in the order of HB^+, BMS, HB^-. This provides evidence that the maintenance of ATP levels brought about by the presence of haemoglobin was not the result of electron transport-linked oxidative phosphorylation in mitochondria.

Figure 27. Effect of Antimycin A on oxygen uptake by transgenic and wild type maize cells.

All measurements were performed under conditions of normal atmosphere. Oxygen uptake was measured polarographically with an O_2 electrode. Oxygen uptake of Antimycin A (0.2 mM)-treated cells was measured 30 min after the treatment started. Results are expressed as nmol $O_2 \cdot min^{-1} \cdot g^{-1}$ fw cells. Line bars represent SE (n=3).


700
600
500
400
300
200
100
0
Oxygen uptake
Wild
HB+
HB-
Air
Air + Antimycin A

CO_2 evolution is lower in HB^+ cells

Production of CO_2 by a cell may be an indication of its respiratory activity. Earlier experiments may suggest that neither mitochondrial oxidative phosphorylation (Fig. 26), nor the fermentative pathways (Fig. 24; Fig. 25) are the haemoglobin- related source of ATP. Therefore, other possibilities ought to be examined. One could hypothesize that the TCA cycle, powered by the activity of an alternative oxidase in the presence of haemoglobin generates ATP through a substrate level phosphorylation (succinyl-CoA → succinate). A complete oxidation of substrate, in the HB+ cells, with only substrate level phosphorylation would result in increased CO_2 production.

An examination of the CO_2 release under a nitrogen gas atmosphere by the three cell types, shows that, while the wild type and HB^- cells release the same amounts of CO_2, its production in HB^+ cells is 20 to 30% lower (Fig. 28). The lower CO_2 release by HB^+ cells in conjunction with lower ADH activity (Fig. 24), suggesting the possibility of reduced ethanolic fermentation.

Cell viability under hypoxia

The observed differences in energy status and metabolic activities between the three cell lines, could be due to differences in cell survival under stress conditions. Cell viability of the BMS, HB^+ and HB^- lines was assayed with the use of FCR test (Table 8).

Figure 28. CO_2 evolution by maize cells cultured under nitrogen atmosphere.

Results are expressed as mg CO_2 evolved per g fresh weight of cells ± SE (n = 3).

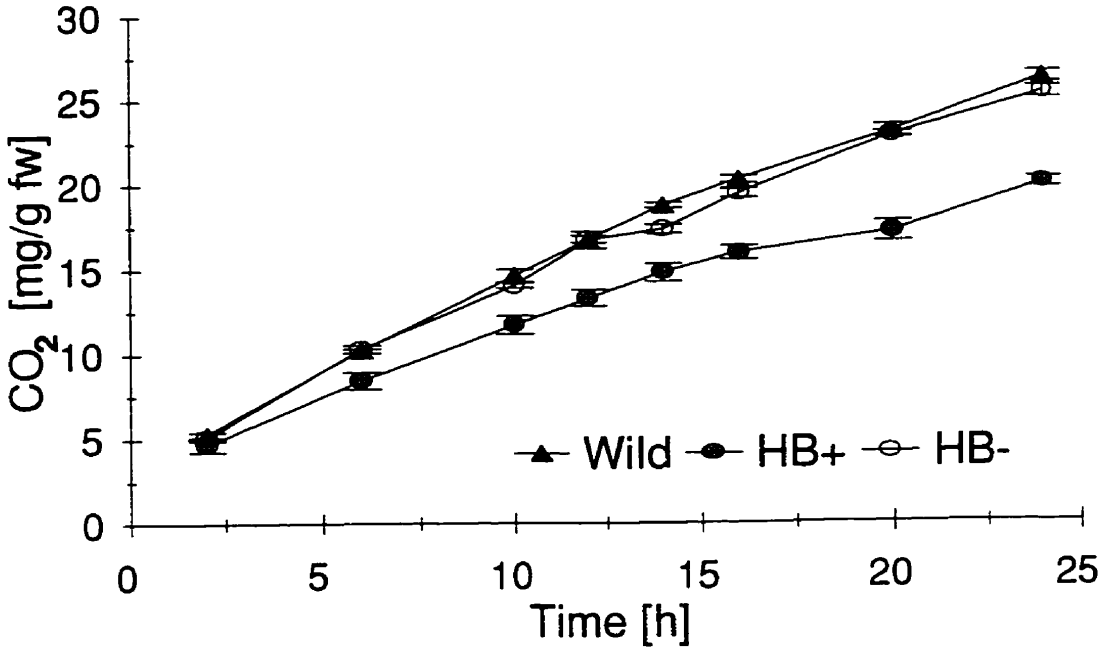

30
25
20
15
10
5
0
CO_2 [mg/g fw]
0
5
10
15
20
25
Time [h]
Wild
HB+
HB-

Table 8. ***Cell viability before and after exposure to nitrogen atmosphere.***

Viability was assayed by FCR test. 300 cells of each sample were observed. Results are means of three experiments. Maximum SE (n=3) was 6%.

	HB-		Wild		HB+	
	Viable cells	%	Viable cells	%	Viable cells	%
Air	283	94	275	91	289	96
12 h N_2	272	90	288	96	272	90
24 h N_2	267	89	254	84	276	90

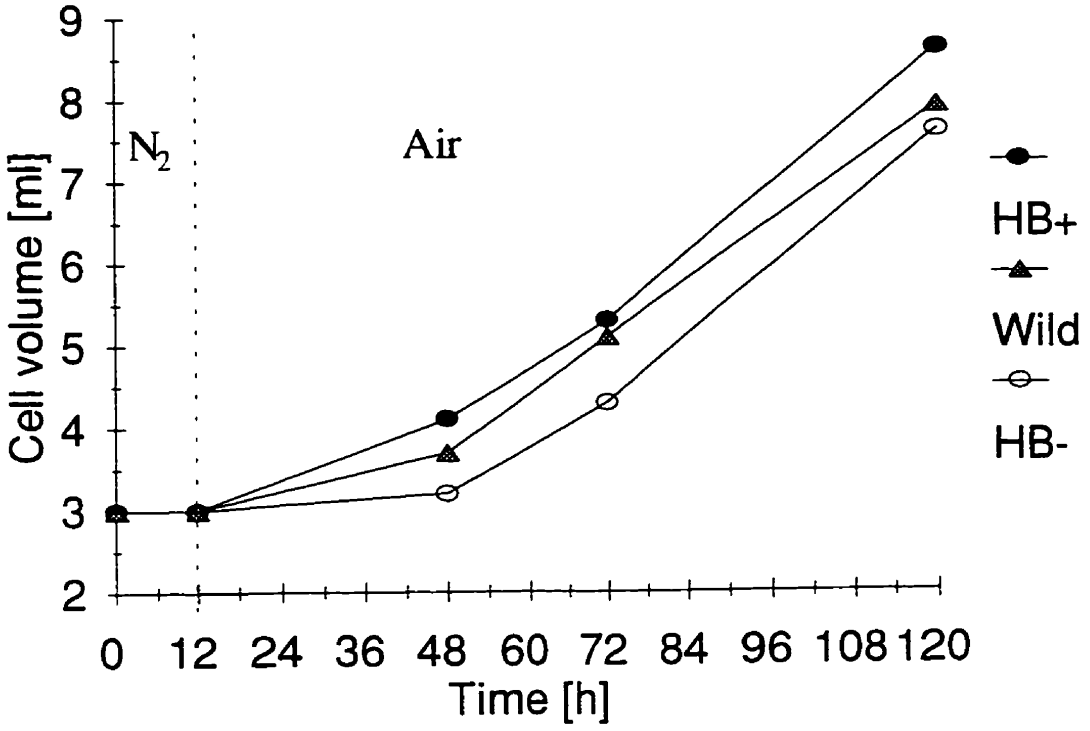
Under normal atmospheric conditions, and after 12 and 24 hours of exposure to nitrogen atmosphere, cell viability was about 90% for all three cell lines. This suggests that the differences in the energy status of cells under hypoxic stress was due to the different metabolic activities rather than to cell death.

The ability of the cell cultures to continue growth after exposure to hypoxic stress was also tested. Maize cell cultures were placed under a nitrogen atmosphere for 12 and 24 hours, then cells were harvested, transferred to a fresh medium and their growth was monitored by sedimented cell volume measurements (Fig. 29). Upon placement under the N_2 atmosphere, the cell growth of all three cell lines ceased, but resumed after transfer to the fresh medium and normal atmospheric conditions. However, while the HB^+ cell cultures resumed growth almost immediately after the transfer to normal air conditions, the HB^- cells showed a 36 hour lag period before commencement of intensive growth. The growth of the wild type cultures, during the first 36 hour after the transfer to normal conditions, was slower than that of HB^+ cells (Fig. 29). After the initial 36 hour period, the growth curves of the three cell lines were almost parallel. The differences in cell volume at each time point were most likely a result of the growth activity during this initial period.

The culture regrowth after the 24 hour hypoxic exposure was the same for all three cell lines, as after the 12 hour treatment (data not shown).

Figure 29. Cell culture growth after hypoxic treatment.

Cultures were placed in the atmosphere of N_2 for 12 hours, then transferred to a fresh medium and continued under normal atmospheric conditions. Cell volume of cultures was measured at indicated time points. SE of all measurements was less than 4% (n=3).


9
8
7
6
5
4
3
2
Cell volume [ml]
N2
Air
HB+
Wild
HB-
0 12 24 36 48 60 72 84 96 108 120
Time [h]

The observed differences could be explained by different levels of cell survival under stress, and depending on the cell line the same cell volume could contain different numbers of growing cells. On the other hand, the steep growth of the HB^- and the wild type BMS cultures (Fig. 29) after a lag period, would suggest a longer stress recovery period rather than cell death. The latter hypothesis is supported by the data on cell viability (Table 8).

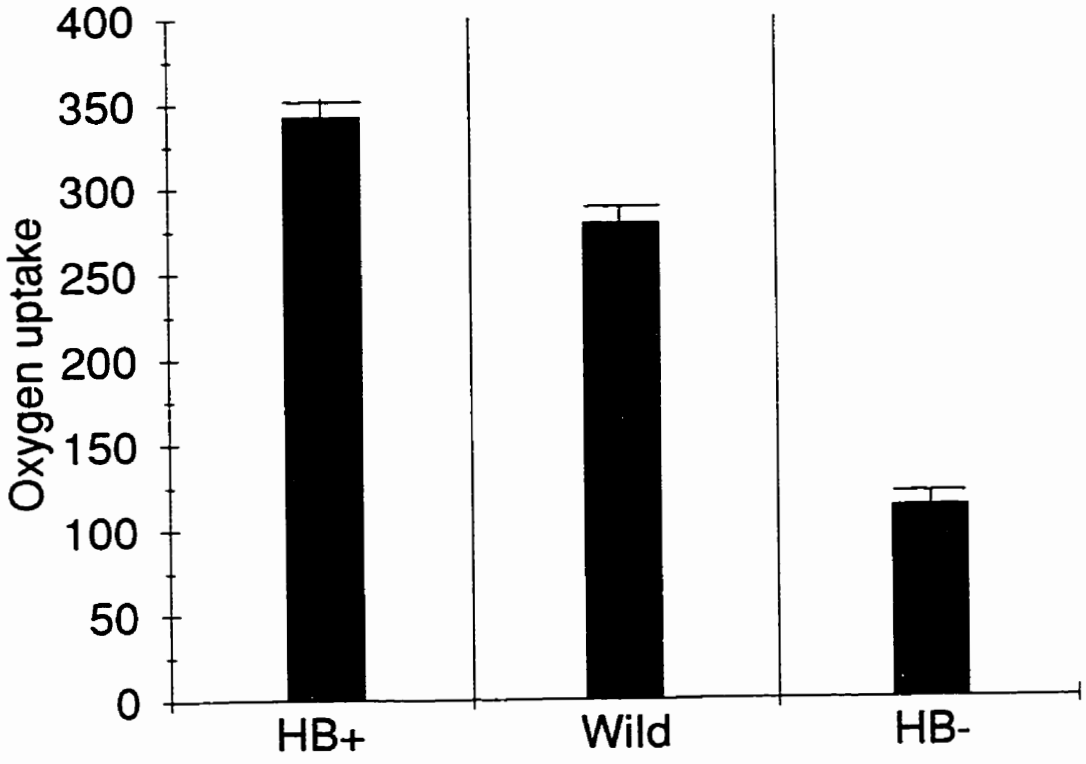
Nonsymbiotic haemoglobin affects oxygen uptake under low oxygen atmosphere

The above presented experiments demonstrate the effects of the presence of haemoglobin on the energy status of maize cells under hypoxia. Differences between the HB^+, wild type and HB^- cells were observed only under conditions of limited oxygen. To investigate the possibility that the observed differences may be due to the different abilities of the cell lines to utilize oxygen that is available in low concentrations, oxygen uptake by maize cells was measured in the medium equilibrated with a mixture of 2% O_2 and 98% N_2 (Fig. 30).

The oxygen uptake rate by HB^+ cells from the medium equilibrated with 2% oxygen, was 45% lower than that of all three cell lines grown under normal air conditions. Wild type BMS cells exhibited a further 20% decrease in the O_2 uptake rate, whereas the oxygen uptake by the HB^- cells declined by a further 65% (Fig. 30). These results indicate that the presence of nonsymbiotic haemoglobin in the maize cells improves the cell's ability to utilize O_2 at low O_2 tensions.

Figure 30. Oxygen uptake by maize cells, under low oxygen atmosphere.

Oxygen uptake was measured polarographically with an O_2 electrode. Cells were placed in medium equilibrated with 98% N_2, 2% O_2 gas. Oxygen uptake is expressed as nmol O_2 · min^{-1} · g^{-1} fw cells. Line bars indicate SE (n=3).


Oxygen uptake
400
350
300
250
200
150
100
50
0
HB+
Wild
HB-

Summary

The wild type BMS, and two transgenic maize cell lines transformed with the sense and antisense barley haemoglobin gene constructs were used in a series of experiments in search of the effects of haemoglobin expression in plant cells. The expression of haemoglobin in the transgenic cell lines was altered to the levels of 10 fold higher (HB^+) and 10 fold lower (HB^-) than that of the wild type BMS cells (Fig. 16).

Expression of haemoglobin, at the levels represented by the HB^+, HB^-, and wild type BMS maize cells, does not affect cell growth or their oxygen uptake (Fig. 22; Fig. 23) under the condition of normal air atmosphere. Similarly, under the same conditions, the activities of fermentative enzymes, ADH and LDH, as well as the energy status of cells, are not affected by the presence of haemoglobin (Figs. 24, 25, 26; Table 7).

The effects of haemoglobin expression were observed, however, under the conditions of limited oxygen supply. Induction of alcohol dehydrogenase (ADH), a typical anaerobic response, was to a lesser extent in the high level haemoglobin expressing HB^+ cells (Fig. 24). Activity of lactate dehydrogenase (LDH) was 10 to 50 times lower than that of ADH in all cell types, suggesting the low contribution of the LDH to the oxygen shortage response. The CO_2 production, under a nitrogen atmosphere, was lower in the HB^+ cells (Fig. 28), reflecting their lower ADH activity. The steady state ATP levels and the energy charge in maize cells, after 12 hours exposure to the atmosphere of pure nitrogen, correlated positively with their haemoglobin content (Fig. 26, Table 7). Neither the ATP level nor the energy charge of the HB^+ cells were affected by the 12 hours of hypoxic exposure. While the wild type cells maintained their energy charge at the cost of

lower ATP level, both the energy charge and the ATP content of the HB^- cells were drastically lowered after the hypoxic exposure (Fig. 26).

When mitochondrial electron transport is blocked by antimycin A, the ATP content of the cells is the same as after the hypoxic exposure (Fig. 26), suggesting that the higher energy status of cells with higher haemoglobin content is independent of the mitochondrial cytochrome electron transport pathway. Effectiveness of antimycin A blockage of the electron transport chain was shown by the 80% decrease in oxygen uptake by all three cell types.

Although the FCR cell viability test (Table 8) did not indicate any substantial cell death rate in any of the three cell lines during the N_2 atmosphere treatment, the culture regrowth rates after the treatment correlated positively with the haemoglobin content of the cells (Fig. 29). The lag period observed in the HB^- cells suggests a longer stress recovery.

The oxygen uptake from the medium equilibrated with 2% oxygen, also correlated with the haemoglobin content of the cells (Fig. 30). The wild type cells utilized oxygen at a rate 20% lower than the HB^+, while in the HB^- cells this rate was 66% lower.

FUTURE WORK DIRECTION

Experiments presented in this section were not repeated, therefore the results should be treated with caution. They might be, however, useful in the further pursuit of the mechanism by which haemoglobin affects the energy status of maize cells under hypoxic stress. Neither ethanolic fermentation (Fig.24), nor mitochondrial oxidative phosphorylation (Fig. 26) are likely to be the pathways by which the improved energy status of cells under hypoxia is achieved in the presence of haemoglobin. Some information about the haemoglobin-related source of ATP, if it in fact exists, might be learned by tracing the fate of substrate in the cells under normal air and hypoxic conditions.

Cell cultures were grown for 10 hours under an atmosphere of air or nitrogen, then transferred to media containing C^{14} labelled sucrose, and the treatments continued for one hour. The total amounts of C^{14} label incorporated by the cells under normal air conditions was similar for all three cell types (Table 9). Under the conditions of limiting oxygen the total label doubled in the HB^+ and wild type cells, but decreased by half in the HB^- cells. This increase in the HB^+ and wild type cells was attributed to the increase in the neutral fraction (mainly sugars). The acidic fraction (mainly organic acids and sugar phosphates) was the source of the label decrease in the HB^- cells (Table 9). The basic fraction (mostly amino acids), in all cell types and treatments was labelled only marginally. These results may suggest that in maize cells experiencing conditions of limiting oxygen the presence of the nonsymbiotic haemoglobin shifts metabolism towards production of neutral metabolites or decreases the rates of sucrose utilization.

Table 9. ***C^{14} labelled metabolites of transgenic and wild type maize cells.***

C^{14} labelled sucrose was added to normally grown and 10 hours hypoxia treated cell cultures. Labelling continued for 1 hour. Cell extracts were separated into neutral (mainly sugars), acidic (mainly organic acids and sugar phosphates), and basic (mainly amino acids) fractions by ion exchange chromatography using Dowex 1 X10, and Dowex 50 X8 columns.

	HB^+		Wild		HB^-	
Treatment	Air	N_2	Air	N_2	Air	N_2
Crude extract[a]	1172292	2003551	968372	197312	1496183	699536
Neutral[b]	28 %	57 %	21 %	51 %	18 %	38 %
Acidic[b]	71 %	42 %	78 %	48 %	81 %	61 %
Basic[b]	0.5 %	0.2 %	0.3 %	0.3 %	0.4 %	0.3 %
Total recovered[a]	1075496	1763074	928449	178467	1351572	648034

[a] total radioactivity in disintegrations per minute (dpm) per g fresh weight

[b] fraction of the total recovered radiactivity

Results indicate an accumulation of neutral products in the HB^+ cells, and negatively charged products in the wild type cells, as well as a decrease in the negatively charged products in the HB^- cells, under the limiting oxygen stress.

The acidic fractions were further separated with the use of a Aminex HPX-78H reverse phase HPLC column. The specific and total activity of the recovered peaks is presented in Table 10. Hypoxic exposure resulted in significant changes in production of acidic metabolites and specific activity of several peaks in different cell types (Table 10). For example, the peak with retention time of 9.31 min is formed only under limiting oxygen conditions. HB^+ cells, under hypoxia, showed a prominent decrease in both total and specific activities of peaks with retention times of 7.05, 15.22, and 22.7 min, whereas in HB^- decrease was observed in 10.6, 13.6, 19.5 min peaks. Peaks with retention times of 6.7, 9.07, 9.69, and 14.7 min appear to be associated with normal air metabolism. However, these changes in specific activity, in any of the peak fractions, did not follow the haemoglobin content of the cell types. Additionally, the identification of these peaks is difficult, since their retention times do not match any of the organic acids or sugar phosphate standards.

Table 10. ***Separation of the C^{14} labelled acidic fractions of cell metabolites.***

Acidic cell metabolite fractions (Table. 9) were further separated by HPLC, using an Aminex HPX 87-H column. Total (TA) and specific (SA) activities are in relative values. Peaks are designated by their retention time (RT).

	HB+				Wild				HB-			
	AIR		N_2		AIR		N_2		AIR		N_2	
RT	SA	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA	TA
6.7	20.4	6.79			0.5	0.54			0.68	0.28	1.4	28.6
7.05	1.36	22.6	0.16	2.11	3.6	53.9	0.23	2.7				
7.3									0.23	2.4	4.35	1.58
9.07	1.98	4.68			1	8.72						
9.31			8.2	127			9.6	257			4.05	38.2
9.69	3.19	5.69			2.6	3.07			4.4	5.9	1.5	6.14
10.6	1.35	7.15	3.93	7.86	3.1	10.4			20.4	53.8	2.5	3.94
11.2	1.5	8.21	2.1	9.93	1.2	3.55	4.84	20.2			2.5	11.6
11.4					1.21	5.14			4.4	14.1		
12.4	5.09	6.53	9.01	3.52					6.5		7.5	5.03
12.9	3.44	4.05	3.77	5.56	1.09	1.99	12.2	19.7	2.5	9.26	3.6	5.78
13.6	2.23	6.99	2.57	5.23	1.02	3.46	5.3	11.2	40	13.3	1.4	4.21
14.7	19	53.8			0.84	2.8	3.5	9	9.3	4.08		

	HB+				Wild				HB-			
	AIR		N_2		AIR		N_2		AIR		N_2	
RT	SA	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA	TA
15.2	38.9	61.8	0.69	7.71	5.42	21.1	34.6	78.6	8.6	155	1.91	169
16.8	0.38	5.96	2.14	9.57	1.27	4.95	8.2	16.3	4.4	7.9	3.09	4.36
18.6			11.8	3.7	6.68	3.11	11.7	4.56	5	1.3	4	2.07
19.5	8.99	5.83	3.25	2.29	5.15	2.92	8.2	7.24	80.6	5.6	2.1	2.81
21.6			4.75	1.76					4.6	3.22	7.5	1.13
22.7	43.3	14.3	1.5	2.33	9.04	7.89	22.6	13.5		27.1	1.4	2.51

V. DISCUSSION

Transformation of maize cells

The main purpose of this study was to demonstrate the physiological significance of nonsymbiotic plant haemoglobin through genetic manipulation of Hb expression. The work presented here is based on the use of the nonsymbiotic haemoglobin gene isolated from barley (Taylor *et al.*, 1994). It was, therefore, essential that the plant material used in this study possesses a nonsymbiotic haemoglobin gene similar in sequence and expression pattern to that of barley. Firstly, if there is any interaction between haemoglobin and any other component of the cell or if the activity of haemoglobin is affected by the environment of the cell, the transgene haemoglobin should have a similar structure and similar properties to the native Hb of the cell in order to be recognized and function properly. Secondly, since the antisense RNA-mediated gene silencing is homology dependent, the nucleotide sequence homology between the transgene and the native Hb genes should be high enough to grant cross-hybridization for the success of the antisense transformation. Finally, for the reason of experimental detection, the native Hb protein, gene and transcripts should be detectable with the available barley Hb antibodies and cDNA probes. All of these criteria have been fulfilled and this is discussed in later sections.

Transformation of the cell, as well as plant regeneration of the monocotyledonous species is usually more difficult to obtain than in dicots (Potrykus, 1991). Although good monocotyledonous plant transformation systems are available, with the particle gun-

mediated DNA delivery being the most successful (Sanford *et al.*, 1987; Gordon Kamm *et al.*, 1990; Fromm *et al.*, 1990), they all require specialized equipment that is not available in most laboratories. For this reason the silicon carbide fibres-mediated transformation of nonregenerative maize suspension cells was selected for use in this study. The method has been used for transient and stable transformation of maize cells (Kaeppler *et al.*, 1990; Kaeppler *et al.*, 1992). Frame *et al.* (1994) regenerated maize plants from maize suspension cells transformed with the use of silicon carbide fibres. Results of the DNA delivery and efficiency of stable transformation, reported by these authors, differ greatly. Kaeppler *et al.* (1992) reported 40 GUS expression units, as assayed by transient histochemical staining, and 3.4 stably transformed colonies per treatment of 300 μl of maize cells. The use of osmotic pretreatment (Frame *et al.*, 1994) resulted in a great increase in the efficiency of DNA delivery. Although as much as 700 GUS expression units were achieved with the use of osmotic pretreatment, efficiency of stable transformation was only 0.17 colonies per treatment (Frame *et al.*, 1994). In the experiments presented in this thesis, the silicon carbide fibres- mediated DNA delivery was adjusted to achieve over 80 GUS expression units per treatment (Figs 10; 11). This gave a mean of 1.55 stably transformed colonies per treatment (Table 2). The efficiency of the method is sensitive to the conditions and duration of the silicon carbide fibres treatment (Fig. 10). Frame *et al.* (1994) reported an almost three fold increase in the DNA delivery by using a different type of vortex mixer. Thus results of the three very similar experiments differ significantly, suggesting that the technique should be tested and adjusted for each cell culture and conditions of the laboratory.

Southern blot and PCR analyses of genomic DNAs were used to examine haemoglobin gene constructs introduced to the genome of maize (BMS) cells. Both analyses show correct band sizes, as expected from plasmid vector DNA, indicating that no DNA rearrangement, in the region of the Hb gene and adjacent Ubi1 promoter, occurred during or after transformation (Figs. 8; 13; 14).

During selection of stable transformants glufosinate ammonium, phosphinothricin or bialophos are usually used in concentrations ranging from 0.5 to 6 mg/l. In experiments presented here, a concentration of 5 mg/l was used. This was almost 2 and 6 times higher than that used by Kaeppler *et al.* (1992) and Frame *at al.* (1994) respectively, yet only about 50% of selected cell colonies proved to be stably transformed as assayed by Southern blot analysis and PCR (Table 2). This indicates that a higher concentration of glufosinate ammonium may be necessary. In the experiment presented here, all cell colonies that survived selection pressure, regardless of their growth rates, were collected and analysed. The selection of only the fastest growing colonies would, most probably, increase the effectiveness of selection and reduce the amount of work related to genetic analysis of selected colonies. Finally, the silicon carbide fibres-mediated genetic transformation of maize cells is an effective and reliable method, in both transient and stable transformation systems, that can be used in virtually any laboratory.

Haemoglobins of maize and barley

Barley Hb cDNA cross-hybridizes with genomes of other monocotyledonous plant of genera *Triticum*, *Secale*, *Zea* and *Triticosecale* (Taylor *et al.*, 1994). Results presented

in this thesis show similarities between the barley and maize haemoglobins. The sequence homology between both genes appears to be high enough for the detection of maize Hb genomic sequence and transcripts with the barley Hb cDNA probe in Southern and Northern blot analyses (Figs. 4; 5). The pattern of expression of both genes is the same. Both Hb genes are induced under condition of oxygen shortage (N_2 atmosphere), showing the same induction pattern with the expression being highest at 12 hours of stress and beginning to decline after 24 hours (Taylor *et al.*, 1994; Fig. 5). Some other results presented here may however, indicate differences between the nucleotide sequences of barley and maize Hb genes. Southern blot analysis of genomic DNA of various plant species (Taylor *et al.*, 1994) using the barley Hb cDNA probe, shows stronger hybridization signals from barley than from maize. Similar Southern analyses of transformed maize cells, presented here, show that the transgene hybridization bands appear stronger than those of the native maize Hb gene (Fig. 13). In most of the cell lines, the 10 kb *PstI* fragment that is expected of the native maize Hb gene (Fig. 4) is absent from Southern blots showing the presence of transgene Hb (Fig. 13) due to the different levels of exposure needed to visualize the two genes. Also the transgene Hb hybridization bands vary in strength between cell lines, suggesting different numbers of copies of the introduced Hb gene.

The HBF and HBR primers used for PCR detection of Hb gene were also used to clone a 1.2 kb genomic Hb sequence from barley (Guy *et al.*, 1997). These primers failed to amplify a genomic fragment from the maize Hb gene (Fig. 14). The HBF and HBR primers anneal to the 5' and 3' ends of the barley Hb cDNA and fall outside of the regions

conserved among plant haemoglobins (Taylor *et al.*, 1994; Andersson *et al.*, 1996) and so are barley specific.

The low frequency of the antisense transformation in suppressing the expression of the native maize Hb gene may also indicate some sequence differences between barley and maize genes (Table 3). Antisense RNA-mediated gene silencing is a homology dependent phenomenon (Izant and Weintraub, 1984; Sandler *et al.*, 1988; Krol *et al.*, 1988), yet a full homology between sense and antisense genes is not necessary for successful silencing. A high frequency inhibition of a tobacco chalcone synthase (CHS) gene was achieved using a 80% homologous antisense chs gene from *petunia* (Krol *et al.*, 1988). The effect of haemoglobin expression on energy status of cells under low oxygen stress, described in this thesis, (Fig. 26; Table 7) may offer an alternative explanation for the low frequency of antisense RNA silencing of Hb expression amongst the pAS2 transformants. Transgenic cell colonies are selected on a solidified medium in sealed Petri dishes. Oxygen conditions during the selection are, therefore, expected to be poor, and cells with a strong suppression of haemoglobin may not be able to survive the eleven week selection process.

Cosuppression is often described in plants transformed with a sense gene construct that is homologous to one of the genes already existing in the plant's genome. This phenomenon has been reported to occur with frequencies of 42% (Krol *et al.*, 1990b). Among the 24 Hb sense (pAS1) transformed maize cell lines inhibition of haemoglobin expression was not observed. In fact they all exhibited highly elevated levels of haemoglobin. The lack of cosuppression amongst the sense transformants may also suggests a lower sequence homology between the barley and maize Hb genes. It should be

noted, however, that 24 transformed cell lines is a relatively low number to make this conclusion statistically probable. Also the possibility of negative selection of cells with a strong suppression of haemoglobin gene expression, as in the case of the antisense transformants, can not be ruled out.

The questions concerning the extent of homology between barley and maize haemoglobin genes can only be fully answered by sequencing of the maize Hb gene. Recently, small amounts of maize haemoglobin were purified by chromatographic methods followed by SDS-PAGE (Guy, Sowa & Hill, unpublished). This protein will be N-terminally and internally sequenced, and these protein derived sequences will be used to generate primers for PCR cloning of the maize Hb gene.

HB^+, wild type, and HB^- - a transgenic system for haemoglobin studies

The wild type BMS maize cells possess, like barley, a single copy nonsymbiotic haemoglobin gene (Fig. 4). This gene is hypoxia-inducible in the suspension-cultured BMS cells (Fig. 5), and the pattern of its induction is similar to that in barley aleurone and root tissues (Taylor *et al.*, 1994), and maize roots (Silva, 1997). Therefore, the BMS cell suspension cultures serve as a good experimental system as well as the aleurone or root tissues. There are, however certain advantages in using the cell suspension cultures. First of all, regeneration of transgenic plants is not needed to conduct the studies. Secondly, the cell suspension culture provides a uniform tissue that is easy to collect and process so that cross-tissue contamination and dissection from the plant are not a problem. Each cell of the culture is an exact clone of a single transformed cell. Since only somatic reproduction

is involved in the continuity of suspension cell cultures, the risk of genetic recombination and subsequent loss of the transgene is very low. This is a serious problem with sexually reproducing transgenic plants.

The HB^+, wild type, and HB^- maize cell lines represent three different levels of haemoglobin expression. Western blot analysis of the total soluble protein isolated from these three cell types, grown under normal air conditions, and its densitometric comparison with a standard curve of known amounts of haemoglobin indicates that the transformation with sense and antisense Hb constructs resulted in a 10 fold increase and decrease respectively in haemoglobin expression over the levels present in the wild type cells (Fig. 16). It should be noted here that the antibodies used in this study were raised and titrated against recombinant barley Hb. Therefore, a possibility exists that these antibodies may be less accurate in detecting the haemoglobin of maize. If so, haemoglobin concentration in all three cell lines may be underestimated. In fact, the protein immunoblot analysis shows no change in haemoglobin levels in all three cell lines after a 24 hour hypoxic treatment (Fig. 18). Northern blot analysis, on the other hand shows a strong induction of the Hb transcript in wild type cells under the same conditions (Fig. 5). This may indicate that unless a strong translational regulation exists, the protein immunoblot detection of maize haemoglobin is less sensitive than Northern blot. The Western blot analyses, however, show a clear difference in haemoglobin content of these three cell lines (Figs. 16; 18), and if the levels of Hb in the wild type and HB^- cells are underestimated so are the differences between them.

Transformation resulted not only in altered levels of haemoglobin polypeptide, but in fully assembled haem-bound protein as was shown by purification of haemoglobin from the HB^+ and wild type cells (Tables 4; 5; Figs. 19; 20). The differences are visualized by the A_{412} readings, even at the beginning of the purification process (Tables 4; 5). Haemoglobin purification from the wild type (BMS) cells was not effective due to the low amounts of Hb present. In the HB- cells haemoglobin was undetectable by Western blot or A_{412} readings.

Haemoglobin isolated from the HB^+ cells shows the same spectral characteristics as the recombinant haemoglobin of barley (Table 6; Duff *et al.*, 1997). This recombinant barley Hb has been expressed in *E.coli* using the *lacZ* operon and has 5 added and 2 changed amino acids at the N-terminus (Duff *et al.*, 1997). The transgene haemoglobin of the HB^+ cells is identical to that of the native barley Hb. These two proteins share identical spectral characteristics in both oxygenated and deoxygenated forms, down to the split of the α band into 528 and 534 nm, and the β band into 554 and 562 nm peaks. This can only reinforce the validity of spectral characteristics and ligand combination rates obtained with the barley recombinant Hb (Duff *et al.*, 1997).

Effects of haemoglobin expression on the energy status of maize cells under hypoxia

The lack of effect of haemoglobin on cell growth and oxygen uptake under normal air conditions likely reflects the fact that barley and maize haemoglobin genes are effective under conditions of limited oxygen availability, with the protein having little effect when oxygen supplies are not limiting. The energy status of maize cells when oxygen is limiting,

however, is effected by the ability of the cells to produce haemoglobin. Total adenylates and ATP levels are maintained during the period of exposure to limiting oxygen, when haemoglobin is constitutively expressed in the cells. Alternatively, when haemoglobin expression is suppressed by constitutive expression of antisense barley haemoglobin message, the cells are unable to maintain their energy status during oxygen limitation. In wild type (BMS) cells it would appear that the induction of native maize haemoglobin is sufficient to maintain energy charge, but not the total adenylate pool. A decline in adenylate pools has been observed during anoxia in maize (Saint Ges *et al.*, 1991) and pea root tips (Andreev *et al.*, 1991). A study of oxygen-limited metabolism of *E.coli* expressing *Vitreoscilla* haemoglobin (VHb), suggested that the expression of VHb resulted in an increased efficiency of ATP production (Khosla *et al.*, 1990). This suggestion is in agreement with the results presented here.

Under limiting oxygen, plants turn their metabolism towards fermentation in order to oxidise NADH, a process that is necessary to maintain glycolytic substrate phosphorylation. In the maize (BMS) suspension cultured cells ethanolic fermentation seems to be the main route since the activities of alcohol dehydrogenase (ADH) are 30 to 80 times higher than those of lactate dehydrogenase (LDH) (Figs. 24; 25). The ADH activity in HB^+ cells during limiting oxygen treatment was lower than in the HB^- and wild type cells. This may suggest that haemoglobin provides an alternative to potentially harmful fermentation. Carbon dioxide is produced by the HB^+ cells in lower amounts than by HB^- and wild type maize cells, reflecting lower ADH activity and suggesting that ethanolic fermentation is the main source of CO_2 during hypoxia in these cells (Fig. 28).

The C^{14} labelling experiment may also suggest a lower utilization of sugars in the HB^+ cells during hypoxia.

The dissociation constant of barley oxyhaemoglobin is 3 nM (Duff *et al.*, 1997), indicating that oxyhaemoglobin acting alone would be ineffective in providing oxygen to maintain mitochondrial respiratory processes. This is confirmed by the observation that antimycin A had no effect on the ability of haemoglobin containing cells to maintain their energy status under low oxygen tensions (Fig. 26). Antimycin A was shown to be a potent inhibitor of respiration in all three maize cell types. It inhibited about 80% of oxygen uptake by these cells under normal air atmosphere (Fig 27), leaving about 20% of the uptake probably accounted for by the alternative oxidase pathway. Since the alternative oxidase pathway does not contribute to mitochondrial ATP production, these results suggest that haemoglobin helps maintain the energy status under limited oxygen availability of the cell by means different from mitochondrial oxidative phosphorylation. This is in contrast with the observation that improved energy production in *Saccharomyces cerevisiae* expressing VHb was inhibited by antimycin A (Chen *et al.*, 1994).

Although barley haemoglobin does not seem to support mitochondrial respiration of maize cells it does effect their oxygen uptake under conditions of low oxygen tensions (Fig. 30). This suggests that haemoglobin plays a role in an oxidative process or in oxygenation of a cellular compound. It could also explain the lower ADH activity of the HB^+ cells under hypoxia. It would appear that during hypoxia these cells have more available oxygen than the other two types. A question might be raised whether the

observed differences in oxygen uptake at low oxygen tension between the three cell lines results directly from the presence of haemoglobin, or indirectly through the effect of haemoglobin on the energy status of maize cells. In other words, is the energy status under hypoxia a result of oxygen uptake and utilization or the oxygen uptake a result of the overall state of the cell. The oxygen uptake measurements were conducted almost immediately (within 30 sec.) after the placement of the cells in medium saturated with 2% oxygen. Cell viability should not be effected so quickly, and therefore it would seem that haemoglobin is directly involved in the uptake and utilization of oxygen under such conditions.

Some of the results presented in this thesis, such as the differences between the three cell lines in enzyme activities, ATP content and energy charge, and oxygen uptake could be simply explained by cell death, if they were analysed separately. For instance the lower ADH activity of the HB^+ cells (Fig. 24), or the lower ATP content of the HB^- cells (Fig. 26), or their lower oxygen uptake under oxygen limiting conditions may be a result of the presence of a number of dead cells in the sample under analysis. This explanation is not convincing when these different experimental data are analysed in concert. For example if the lower ADH activity of the HB+ cells results from increased cell death how does one explain their higher energy charge and ATP content or higher rates of oxygen uptake?

It is difficult to pin-point or even define the moment of cell death. The fluorescein diacetate (FCR) staining test, used to assess cell viability is based on the integrity of the plasmalemma Heslop Harrison and Heslop Harrison, 1970; Heslop Harrison *et al.*, 1984)

and may possibly overestimate the cells ability to continue growth and divide. The test, however, did not show any significant differences in cell viability between the three cell lines after 12 hours of hypoxic treatment (Table 8), when the adenylates were assayed. The ability of the cell cultures to continue growth after the treatment with nitrogen atmosphere was also tested (Fig. 29). Results show that regrowth rates of cell cultures correlated with their haemoglobin content. This may indicate that increased cell death rates occur in the wild type and Hb$^-$ cells. The growth lag period followed by an intensive culture growth would rather suggest a longer time required for stress recovery by these cells, perhaps to regain energy status.

Function of the nonsymbiotic haemoglobin

The available evidence suggests that nonsymbiotic haemoglobins are present widely across the plant kingdom. Based on protein sequence comparisons and the common occurrence of the nonsymbiotic haemoglobins in plants, it has been proposed that the nonsymbiotic Hbs appeared early in the evolution of plants (Andersson *et al.*, 1996). Nonsymbiotic haemoglobins are expressed in a number of plant tissues such as roots (Taylor *et al.*, 1994; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997; Andersson *et al.*, 1996), aleurone (Taylor *et al.*, 1994) and vascular tissues of leaves, stems and seedling cotyledons (Andersson *et al.*, 1996). Recently the expression of a nonsymbiotic haemoglobin was observed in germinating seeds and growing coleoptiles (Duff *et al.*, 1998, in press), and hypoxia-stressed leaves of barley (Nie, 1997). This thesis shows expression of haemoglobin in stressed, suspension cultured, undifferentiated cells of maize

(Fig. 5). It would appear that the expression of nonsymbiotic haemoglobin is specific not to the tissue but to the conditions of growth and metabolic activity of the cell. Haemoglobin is likely expressed in either rapidly growing and metabolically active tissues that are under high demand for energy, or in cells under oxygen stress which limits the production of energy. All the above reasons suggest a fundamental role for nonsymbiotic haemoglobins in a process common to all plants and cells.

Strong evidence supports haemoprotein based oxygen sensing in bacterial (Gilles Gonzalez *et al.*, 1991; Gilles Gonzalez and Gonzalez, 1993; Poole *et al.*, 1994a) and mammalian systems (Goldberg *et al.*, 1988). It has been proposed that nonsymbiotic haemoglobins of plants may function as an oxygen sensor to monitor the oxygen level in tissues and, when necessary, to switch the metabolism from an aerobic to an anaerobic pathway (Appleby *et al.*, 1988). According to this hypothesis, a transition from the oxygenated to the deoxygenated state under lowered oxygen concentration results in a changed conformation of haemoglobin, that in turn triggers the anaerobic response (Goldberg *et al.*, 1988). The bacterial haemoprotein oxygen sensors, FixL and Hmp have oxygen dissociation constants of 50 and 2 μM respectively (Poole *et al.*, 1994b; Gilles Gonzalez *et al.*, 1991), whereas the constants of plant nonsymbiotic haemoglobins range from 0.5 to 2.7 nM (Table 1), are three orders of magnitude lower. With so low oxygen dissociation constants plant haemoglobins will remain oxygenated at oxygen concentrations far below those at which anaerobic processes are activated. Furthermore blocking the expression of haemoglobin in HB^- cells did not result in their decreased anaerobic response (Fig. 24), rather the opposite, while elevated expression of Hb in HB^+

cells resulted in lower activity of the fermentative pathway under hypoxic stress. Also the hypoxic induction of haemoglobin in plant tissues suggests that haemoglobin is a part of the anaerobic response rather than a sensor that switches it on. Finally, it has been demonstrated in barley aleurone tissue that the induction of haemoglobin expression is triggered not directly by the lack of oxygen but by the impaired ATP synthesis that is a result of it (Nie and Hill, 1997).

The low oxygen dissociation constant of the nonsymbiotic haemoglobins also challenges another persisting theory that views haemoglobins as facilitators of oxygen diffusion or oxygen stores supporting mitochondrial respiration under limited oxygen tensions. The Km for oxygen of cytochrome c oxidase is approximately 100 nM (Appleby, 1992). Having an oxygen dissociation constant of about 2 nM (Table 1), nonsymbiotic haemoglobins would be completely ineffective in providing oxygen to the mitochondrial respiratory process. No other known oxidases have a Km for O_2 sufficiently low to make use of this oxygen. Barley haemoglobin shows an extraordinarily slow oxygen dissociation ($t_{1/2}$ = 27 s). This would appear to preclude this haemoglobin from oxygen transport or facilitated diffusion involving reversible oxygen dissociation (Duff *et al.*, 1997).

For all the above reasons, unless the high oxygen avidity of plant nonsymbiotic haemoglobins is altered by an association with another molecule its function as an oxygen sensor or facilitator of oxygen diffusion would seem to be impossible.

Similar to the nonsymbiotic haemoglobins of barley, maize, rice and *Arabidopsis* (Taylor *et al.*, 1994; Silva, 1997; Arredondo-Peter *et al.*, 1997; Trevaskis *et al.*, 1997) a

haemoglobin of the bacterium *Vitreoscilla* (VHb) is induced under conditions of limiting oxygen (Wakabayashi *et al.*, 1986; Dikshit *et al.*, 1992). This bacterial haemoglobin enhances growth of transgenic microorganisms such as *E. Coli* and *Saccharomyces cerevisiae* (Khosla and Bailey, 1988; Chen *et al.*, 1994; Kallio *et al.*, 1994), and plants such as *Nicotiana tabaccum* and *Datura* (Holmberg *et al.*, 1997). This invites a comparison with barley haemoglobin and the studies presented here. It was suggested that in transformed *E.coli*, VHb supplies oxygen to terminal oxidases (Kallio *et al.*, 1994). In transformed yeast VHb appears to interfere with the mitochondrial electron transport chain (Chen *et al.*, 1994). In fact about 40% of the VHb expressed in yeast was found in mitochondria. Contrary to that, the nonsymbiotic haemoglobin of barley was found only in the cytoplasm and not in mitochondria (Nie, 1997). Furthermore, this thesis shows that the interaction of barley Hb with mitochondrial respiration is unlikely. The expression of this protein does, however, enhance the cell's oxygen uptake under conditions of limited oxygen supply.

The data presented in this thesis, taken together, support a hypothesis that nonsymbiotic haemoglobins may function as a terminal oxidase or oxygenase in cells exposed to low oxygen tensions. The very low dissociation constant of barley oxyhaemoglobin make it an ideal candidate for sequestering oxygen in low oxygen environments. Interaction with another compound, perhaps a flavoprotein, could create a complex capable of oxidizing NADH, in a manner analogous to the Hmp protein of *E. coli* (Poole *et al.*, 1996). This would provide an efficient means of oxidatively regenerating NAD to support glycolysis, bypassing the fermentative route to ethanol.

The effects of expression of sense and antisense haemoglobin on energy charge are reminiscent of hypoxic acclimation of plant tissues. Maize root tips develop a tolerance to short term anoxia if they have been acclimated by exposure to hypoxic conditions (Johnson *et al.*, 1989). Acclimation is accompanied by increased energy charge resulting from a sustained glycolytic rate compared to non-acclimated root tips (Xia and Saglio, 1992; Xia and Roberts, 1996). Similarly, winter cereals show increased survival to hypoxia caused by ice encasement if they have been acclimated by exposure to hypoxic conditions (Andrews and Pomeroy, 1983). Acclimated plants maintain higher levels of adenylates and ATP during ice encasement as a result of accelerated rates of glycolysis compared to non-acclimated plants (Andrews and Pomeroy, 1989). Maximum induction of barley haemoglobin message occurs within 12 hours exposure to hypoxic conditions (Taylor *et al.*, 1994), which is well within the time interval used for acclimation in the above examples. Furthermore, it has been shown that the expression of haemoglobin is not directly influenced by oxygen usage or availability but is influenced by the availability of ATP in the tissue (Nie and Hill, 1997). It may be, therefore, that the increased survival of plants to anoxia as a result of hypoxic acclimation, is a consequence of haemoglobin gene induction during the acclimation process as a result of declining ATP levels during the acclimation process.

From an evolutionary standpoint it has been suggested that nonsymbiotic haemoglobins represent one of the more ancient forms of plant haemoglobins (Andersson *et al.*, 1996). The results presented here add credence to this idea. Since early life on

earth existed in oxygen-poor environments, the presence of a haemoglobin capable of utilizing oxygen at low oxygen tensions would have provided an evolutionary advantage to an organism. Oxygen produced during photosynthesis and retained as oxyhaemoglobin would provide a source of oxygen to oxidize NADH, maintaining a high glycolytic flux during darkness to provide ATP for cell growth and development.

Future directions

This thesis has shown the effect of barley haemoglobin on the energy status of maize cells under limiting oxygen, as well as some directions for researching the function of the nonsymbiotic haemoglobins in plants. The demonstration of the effect of haemoglobin expression was achieved using barley Hb gene in maize cells, which may suggest that the function of the nonsymbiotic haemoglobin is common to several, if not all plants. However, to validate these results a similar study should be conducted using other plants species. The results presented here suggest that the expression of plant nonsymbiotic haemoglobin may increase the plant's tolerance to limited oxygen stress. In a natural environment this stress can be imposed by flooding. New data, (Duff *et al.*, 1998) show induction of haemoglobin during seed germination which in conjunction with the results presented here may suggest a possible role for haemoglobin in improving the energetics of germinating seed. Construction of transgenic plants (barley or maize) expressing sense and antisense Hb transcripts would enable studies on the role of haemoglobin during germination and flooding tolerance of plants.

More work is also required on the specific mechanism and site of haemoglobin action. Experiments with C^{14} labelled sucrose indicated a possible correlation between Hb and amounts of acidic metabolites, but did not show accumulation of any specific product (Table 9; 10). Some differences have been observed in the neutral metabolites as well, however, the C^{14} label in these metabolites may be experimentally affected by the labelled sucrose that was added to the cell cultures. It should be noted here that there are some other experimental limits to the approach taken. For instance, any volatile metabolite is lost during the extraction and fractionation, since samples are concentrated by evaporation.

Very promising are the effects of haemoglobin in increasing the oxygen uptake of cells under a low oxygen atmosphere (Fig. 30). The lack of antimycin A effect on cell's ATP levels (Fig.26) would suggest that this haemoglobin-mediated oxygen uptake does not involve mitochondrial electron transport. This point might be strengthened, or opened for reinvestigation after measuring the effect of mitochondrial respiration inhibitors on the oxygen uptake in the three cell lines.

VI. Conclusions

1. Overexpression and silencing of haemoglobin was achieved in maize (BMS) suspension-cultured cells through genetic transformation with gene constructs containing barley Hb cDNA in sense and antisense orientations.

2. Using the transgenic maize cells it was shown that the presence of barley haemoglobin in maize cells improves their ATP content and energy charge.

3. Blocking the mitochondrial electron transport chain with antimycin A did not abolish the haemoglobin-mediated effect on cell's energy status, suggesting that haemoglobin is not involved in supporting respiratory processes in mitochondria.

4. Oxygen uptake by the cells at low (2 %) oxygen concentration correlates with the nonsymbiotic barley haemoglobin content of the cell suggesting that the process in which the haemoglobin is involved uses oxygen.

5. The cell's ability to continue growth after low oxygen stress appears to be enhanced by haemoglobin.

6. It is proposed that the nonsymbiotic haemoglobin may function in oxidizing NADH, thus regenerating NAD to support glycolytic ATP synthesis thus bypassing the fermentative route to ethanol.

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Appendix I

SPECIAL MS (10X CONCENTRATE) FOR BMS SUSPENSION CULTURES
per litre

NH_4NO_3	16.5 g		
KNO_3	19.0 g		
$CaCl_2$	4.4 g		
$MgSO_4 \times 7H_2O$	3.7 g		
Ferric versanate (Fe EDTA)	0.4	EDTA 2Na :	372 mg
		$FeSO_4 \times 7H_2O$:	278 mg
L-asparagine	1.5 g		
Micronutrients (stock)		10 ml	
Thiamine	5 mg		
2,4-D (Stock: 0.5 mg/ml)	40 ml		
Sucrose	200 g		

pH 5.8 before use

Micronutrients

H_3BO_3	6.2 g
$MnSO_4 \times H_2O$	16.9 g
$ZnSO_4 \times 7H_2O$	8.6 g
$Na_2MoO_4 \times 2H_2O$	0.25 g
$CuSO_4 \times 5H_2O$	0.025 g
$CoCl_2 \times 6H_2O$	0.025 g

H_2O to make 1000 ml

2,4-D Stock : 100 mg in 40 ml EtOH 95 % + 160 ml H_2O

Appendix II

Buffers and Solutions

TE (pH 8.0)

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

SSC (20 x)

3 M NaCl

0.3 M Na_3 citrate

Denhardt's solution (100x)

2% (w/v) Ficoll

2% (w/v) PVP (Polyvinylpyrrolidone)

2% BSA (bovine serum albumin)

TBS (Tris-buffered saline, pH 7.4)

0.8% (w/v) NaCl

0.02% (w/v) KCl

0.3% (w/v) Tris

0.0015% (w/v) phenol red

TTBS (pH 7.4)

TBS

0.05% Tween 20

CTAB buffer

2% CTAB (cetyltrimethylammonium bromide, w/v)

100 mM Tris (pH 8.0)

20 mM EDTA (pH 8.0)

1.4 M NaCl

1% PVP (polyvinylpyrrolidone)

TAE buffer

0.04 M Tris-Acetate

0.001M EDTA

10 x **RNA gel-running** buffer

0.2 M MOPS (3-(N-morpholino)propanesufonic acid, pH 7.0)

80 mM Na-Acetate

5 mM EDTA (pH 8.0)

Appendix III.

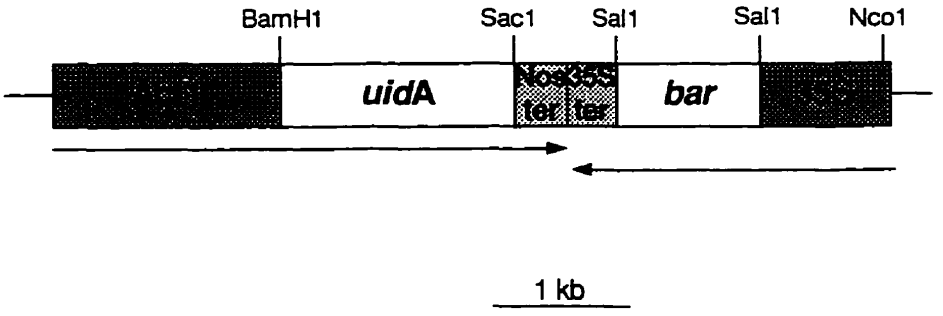
Primer annealing sites in pAS1 and pAS2 plasmid vectors

pAS1

pAS2

Appendix IV

pDB1 plasmid



Act1 - Rice actin 1 gene promoter

35S - CaMV 35S promoter

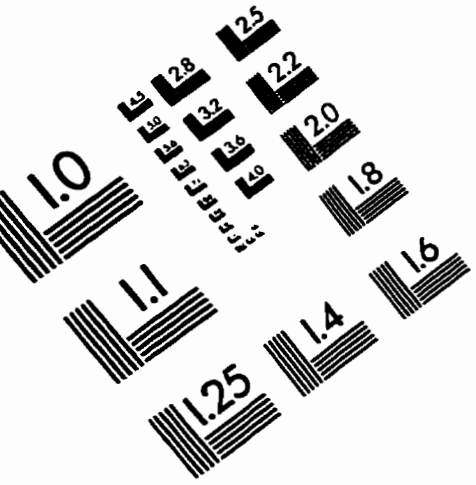
uidA - β - Glucuronidase gene

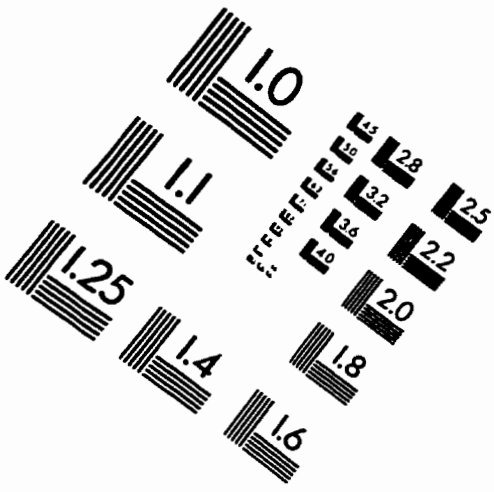
bar - Phosphinothricin acetyl transferase gene

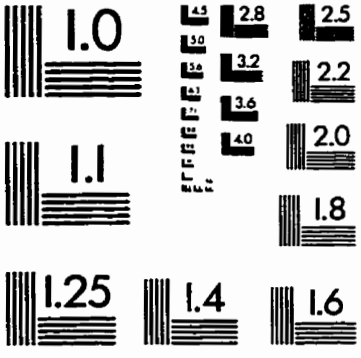
Nos-Ter - Nopaline synthase gene terminator

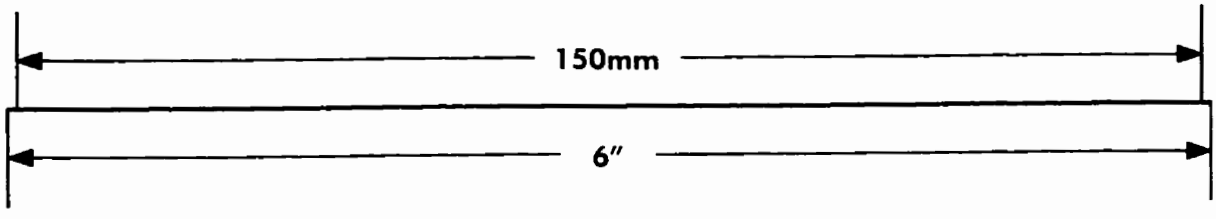
35S-Ter - CaMV 35S terminator

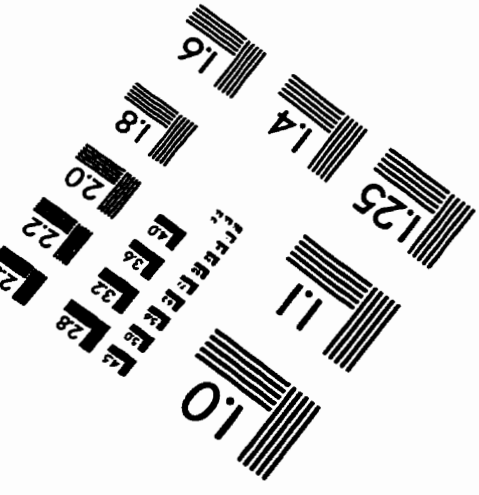
IMAGE EVALUATION
TEST TARGET (QA-3)

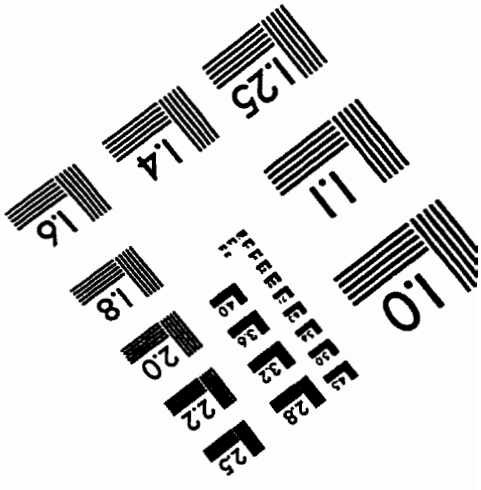












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